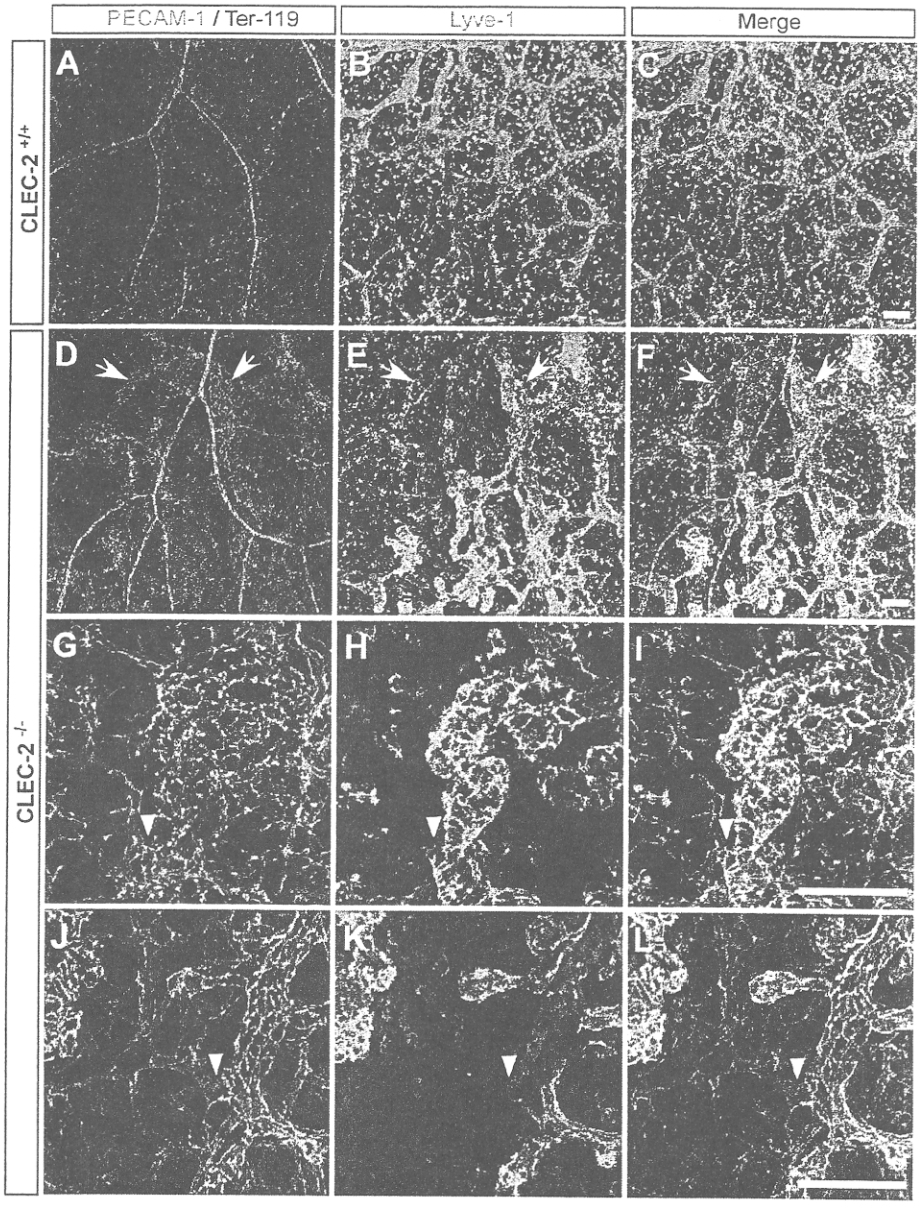


FIGURE 3. **Blood-filled disorganized lymphatic vessels and abnormal connection between blood and lymphatic vessels in *Clec-2*$^{-/-}$ embryos.** Whole-mount triple fluorescence confocal microscopy of embryonic back skin was performed with antibodies to PECAM-1 (*red*), LYVE-1 (*green*), and TER-119 (*blue*) at E14.5 (*A–I*) and E17.5 (*J–L*). *A–F*, whereas blood vessels visualized by PECAM-1 staining appear unaffected in *Clec-2*$^{-/-}$ embryos, lymphatic vessels visualized by LYVE-1 staining are disorganized and distended in *Clec-2*$^{-/-}$ embryos. Lymphatic vessels are filled with TER-119^{+} erythrocytes (*arrows*) in *Clec-2*$^{-/-}$ embryos. *G–L*, abnormal connection sites (*arrowheads*) between blood and lymphatic vessels were detected in *Clec-2*$^{-/-}$ embryos. *Scale bars* = 100 μm.

stained for LYVE-1 and PECAM-1 exhibited a dilated, tortuous, and rugged appearance, which was in contrast to the narrow, straight, and smooth appearance observed in WT embryos (Fig. 3, *A–F*). This analysis also revealed anastomotic sites of blood and lymphatic vessels in CLEC-2-deficient embryos at E14.5 (Fig. 3*I*) and at E17.5 (Fig. 3*L*).

Upon CLEC-2 simulation of platelets with rhodocytin or podoplanin, the tyrosine kinase Src phosphorylates a single YXXL motif in its cytoplasmic domain. The tyrosine kinase Syk then binds to the phosphorylated YXXL motif through its SH2 domains, which leads to Syk activation, followed by tyrosine phosphorylation of the adaptor proteins SLP-76 and LAT (linker for activation of T cells). Phospholipase Cγ2 is finally activated, which results in Ca^{2+} increase and platelet aggregation. We and others have reported previously that Src, Syk, LAT, SLP-76, and phospholipase Cγ2 are necessary for CLEC-2-mediated signal transduction (3, 21, 22). Disorganized and blood-filled lymphatic vessels observed in CLEC-2-deficient embryos were also observed in embryos deficient in Syk or SLP-76 (10) or phospholipase Cγ2 (23). These molecules are also necessary for signal transduction pathways mediated by the collagen receptor GPVI/FcR γ-chain. However, blood-filled lymphatic vessels were not observed in mice deficient in the GPVI/FcR γ-chain but only in CLEC-2-deficient mice, suggesting that platelet activation through CLEC-2, but not through the GPVI/FcR γ-chain, is important for blood/lymphatic vessel separation. We propose that CLEC-2 is essential for blood/lymphatic vessel separation, which is necessary for the survival of murine embryos.

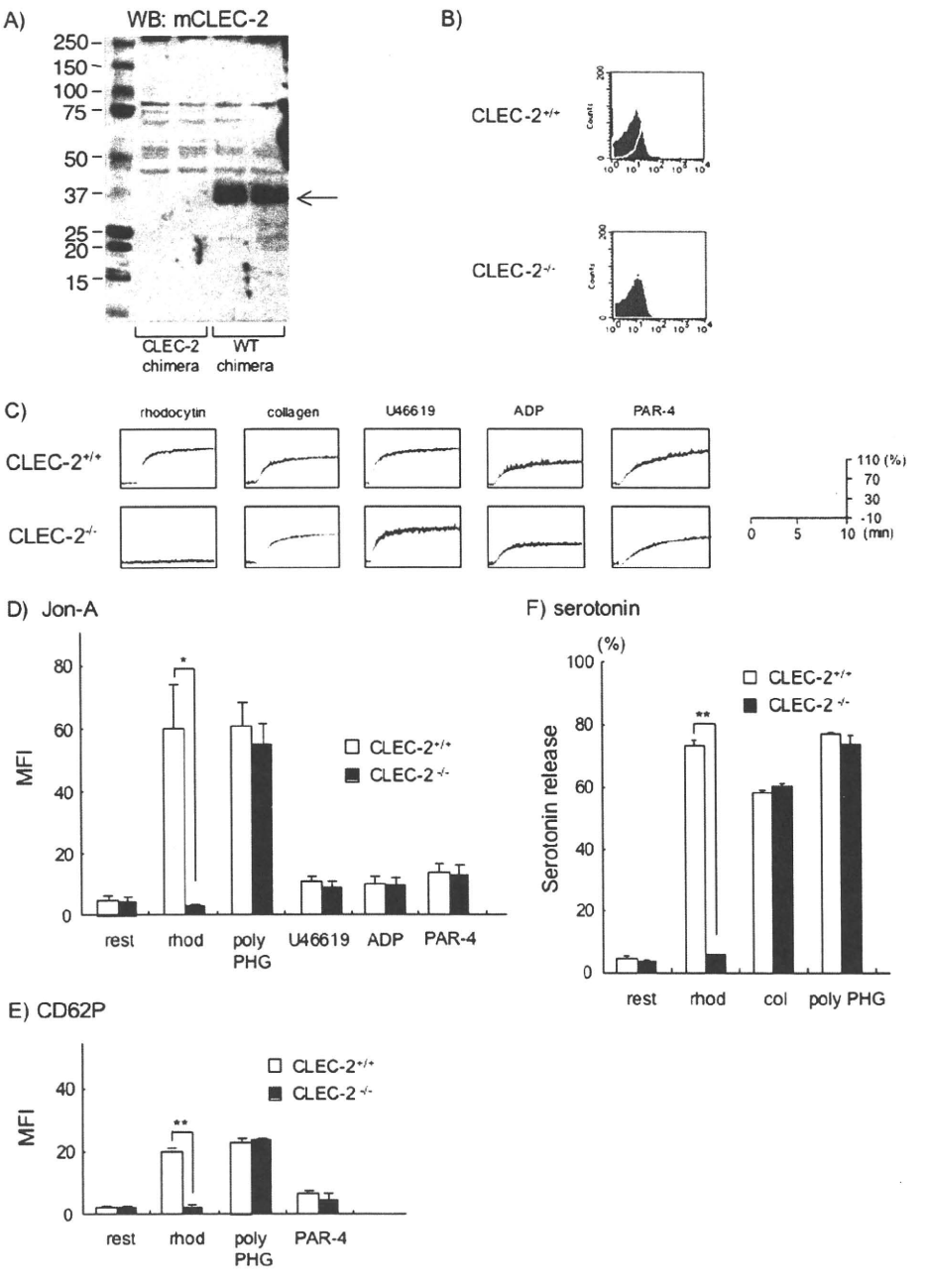
Platelets Deficient in CLEC-2 Lack Rhodocytin-induced Platelet Activation but Show Normal Responses to Other Agonists or Extracellular Matrices—Because *Clec-2*$^{-/-}$ mice exhibited embryonic and neonatal lethality, we produced irradiated chimeric animals that had been rescued by fetal liver transplantation to investigate a role of CLEC-2 in thrombosis and hemostasis. Mice rescued with WT fetal liver are referred as WT chimeras, and those rescued with CLEC-2-deficient fetal liver are referred as CLEC-2 chimeras. Immunoblotting (Fig. 4*A*) and flow cytometry for CLEC-2 (Fig. 4*B*) confirmed successful reconstitution. CLEC-2-deficient platelets expressed major membrane proteins such as integrins αIIbβ3 and α2β1, GPIb/IX/V, and PECAM-1 at levels similar to WT platelets (supplemental Fig. 2).

In the process of physiological thrombus formation, the initial contact of platelets (tethering) to collagen exposed at sites of vessel injury is mediated predominantly by the interaction between platelet GPIb and vWF adhered to collagen, which is

essential under high shear rates. In the next step, GPVI/collagen interactions initiate cellular activation, followed by shifting of integrins to the high affinity state and the release of second-wave agonists, most importantly ADP and thromboxane A_2 (24). Released ADP and thromboxane A_2 amplify integrin activation on adherent platelets and flowing platelets, which results in platelet/platelet interaction through activated integrin αIIbβ3/fibrinogen interaction, leading to thrombus growth. Therefore, we sought to investigate the responses of CLEC-2-deficient platelets to those agonists or extracellular matrices that participate in physiological thrombus formation. CLEC-2-deficient platelets failed to aggregate in response to rhodocytin, a CLEC-2-activating snake venom, but they fully aggregated upon stimulation with other classical agonists, including ADP, U46619, collagen, and poly(PHG) (a GPVI-specific agonist peptide) (16) and thrombin receptor PAR-4-activating peptide (Fig. 4*C*). Flow cytometric analysis of integrin αIIbβ3 activation and P-selectin exposure, a marker of α-granule release, confirmed that CLEC-2 deficiency had no significant effect on platelet activation by ADP, U46619, poly(PHG), and PAR-4, whereas responses to rhodocytin were abolished (Fig. 4, *D* and *E*). Similarly, serotonin release from dense granules induced by rhodocytin, but not by poly(PHG) or collagen, was abolished in CLEC-2-deficient platelets (Fig. 4*F*). Thus, CLEC-2 deficiency results in the specific loss of the CLEC-2-mediated signal transduction pathway in platelets while leaving other pathways fully intact.

Platelets Deficient in CLEC-2 Show Normal Adhesion and Spreading on the Surface of Collagen, Fibrinogen, Laminin, and vWF—It is well known that platelets adhere and spread on the surface of various extracellular matrices such as collagen, fibrinogen, laminin, and vWF through specific receptors (collagen: integrin $\alpha2\beta1$ and GPVI; fibrinogen: integrin αIIbβ3; laminin: integrin $\alpha6$ and GPVI; and vWF: GPIb/IX/V and integrin αIIbβ3). We next investigated the possibility that CLEC-2 is a receptor for these extracellular matrices and supports platelet adhesion and spreading. As shown in Fig. 5*A*, adhesion and spreading of CLEC-2-deficient platelets on the surface of these extracellular matrices were comparable with those of WT platelets. Quantification of platelet adhesion and spreading showed that there was no statistically significance difference between CLEC-2-deficient and WT platelets (Fig. 5*B*). These findings suggest that CLEC-2 is not an activation receptor for these extracellular matrices.

CLEC-2 Is Required for Stable Thrombus Formation under Flow Conditions—Platelet activation at sites of vascular injury under flow conditions is an integrated process involving subendothelial matrices and soluble agonists, including ADP, thromboxane A_2, and thrombin, that supports adhesion and activation. To investigate a role of CLEC-2 in this process, whole blood from CLEC-2 or WT chimeras labeled with 3,3′-dihexyloxacarbocyanine iodide was flowed over collagen-coated surfaces at a high



shear rate (2000 s^{-1}) for 5 min. As shown in Fig. 6 (*A–C*), WT platelets formed large thrombi on the surface of collagen by the end of a 5-min perfusion period, whereas thrombus formation of CLEC-2-deficient platelets was significantly impaired, suggesting that the CLEC-2-dependent process is essential for stable aggregate formation under flow conditions. CLEC-2-deficient platelets showed normal single-cell adhesion on the surface of collagen (Fig. 5), whereas thrombus formation on the surface of collagen under flow conditions was significantly inhibited (Fig. 6, *A–C*). Taken together, these findings suggest that CLEC-2 is required for the piling-up process of platelets that leads to stable thrombus formation, but not for initial adhesion to collagen.

To investigate whether the thrombus instability was based on impaired platelet activation, we performed flow adhesion studies with or without co-infusion of ADP, which is released from activated platelets. Co-infusion of 10 μM ADP into anticoagulated blood just before entrance into the capillary resulted in the formation of stable thrombi both in control and CLEC-2-deficient blood (supplemental Fig. 3), suggesting that CLEC-2 functions as an activation receptor in platelets that is required for stable thrombus formation.

In Vivo Thrombus Formation Is Impaired in CLEC-2 Chimeras in a Laser-induced Injury Model with Minimal Increase in Tail Bleeding—Because platelet activation/aggregation is a major cause of arterial thrombosis, we studied the effects of CLEC-2 deficiency on pathological thrombus formation. We used a direct visual technique that enabled us to evaluate *in vivo* thrombus stability with great temporal and spatial resolution and to characterize the kinetics of CLEC-2-deficient platelets in thrombus formation. This method is based on confocal microscopy, which permits high spatiotemporal resolution of individual platelets under flow conditions in mesenteric capillaries and arterioles (19, 25). With this system, laser irradiation produces reactive oxygen species, which cause injury to the endothelial layer of the vessels. In WT chimeras, after laser-induced injury to mesenteric capillaries, adherent platelets appeared to recruit platelets in the circulation to form platelet aggregates, and the resultant thrombus reduced the vessel lumen diameter and blood flow velocity. Ultimately, the lumen was completely occluded by thrombi. In contrast, CLEC-2-deficient platelets adhered to the vessel wall more loosely than WT platelets, they were frequently washed away by the blood flow, and the platelet aggregates/thrombi never occluded the capillaries (Fig. 7*A*, *panel i*, and supplemental Videos 1 and 2). As a result, the number of CLEC-2-deficient platelets that accumulated at the injured vessel was 35% less than that of WT platelets (Fig. 7*A*, *panel ii*). These findings suggest that CLEC-2 contributes to the stabilization of developing thrombi in the laser-induced injury model. We next evaluated tail bleeding of WT and CLEC-2 chimeras. Although CLEC-2 chimeras had a tendency to have more blood loss from tail bleeding than WT chimeras, it was not significant (mean blood loss ± S.E. of 13.7 ± 1.3 μl in WT chimeras and 34.7 ± 3.2 μl in CLEC-2 chimeras, $p = 0.08$) (Fig. 7*B*). Taken together, these findings suggest that CLEC-2 deficiency causes impaired thrombus growth with minimal increase in bleeding tendency.

CLEC-2 Forms Homophilic Associations—Thrombus formation under flow conditions *in vitro* and *in vivo* was impaired in CLEC-2 chimeras (Figs. 6 and 7), although CLEC-2-deficient platelets normally aggregated upon stimulation with classical agonists other than rhodocytin and showed normal adhesion and spreading on the surface of major extracellular matrices (Figs. 4 and 5). This suggests that the ligands of CLEC-2 may be present in plasma or on the surface of platelets. CLEC-2 is known to bind to podoplanin depending on glycosylation of podoplanin (4). Therefore, we first investigated the possibility of platelet membrane proteins with glycosylation, including CLEC-2 itself, as candidates for CLEC-2 ligands. To prove the interaction between the molecules directly, we utilized surface plasmon resonance (Biacore AB). Recombinant proteins of interest were perfused over the sensor tip coated with hCLEC-2-rFc2 or mCLEC-2-rFc2. Several commercially available recombinant proteins on the surface of platelet membranes, including integrins $\alpha v\beta 3$ and $\alpha 2\beta 1$, CD62P, LIMP-II, thrombospondin-1, and PEAR-1, were evaluated, but we could not observe their association with recombinant CLEC-2 (data not shown). Finally, we evaluated the CLEC-2 binding, and to our surprise, we detected homophilic interactions of hCLEC-2-rFc2 and mCLEC-2-rFc2 (Fig. 8, *A* and *B*, respectively). The sensorgrams at different analyte concentrations were obtained and normalized by subtracting background signals from the CLEC-2-Fc2 surface. The *arrows* indicate the beginning and end of perfusion of an analyte. After perfusion started, the resonance unit, which indicates the binding of analyte (flowing recombinant CLEC-2) to the ligand (coated recombinant CLEC-2), gradually increased, followed by a gradual decrease after the cessation of perfusion, suggesting that both human and mouse CLEC-2 form homophilic association. The interac-

FIGURE 4. **Responses to rhodocytin are abolished in CLEC-2-deficient platelets, but WT platelets respond normally to other agonists.** *A*, shown is a Western blot (*WB*) of washed platelets from two WT chimeras and two CLEC-2 chimeras with anti-mouse CLEC-2 antibody. The *arrow* indicates murine CLEC-2 (*mCLEC-2*). *B*, whole blood from WT and CLEC-2 chimeras was diluted 15-fold with modified Tyrode's buffer. 25 μl of the diluted whole blood was incubated with Cy2-conjugated anti-mouse CLEC-2 antibody or Cy2-conjugated control rabbit IgG for 15 min at room temperature. Reactions were terminated by the addition of 400 μl of PBS, and the samples were then analyzed using a FACScan. *C*, washed platelets from WT or CLEC-2 chimeras were used for aggregation studies. The washed platelets were stimulated by the indicated agonists, and platelet aggregation was monitored by light transmission using a Born aggregometer at 37 °C for 10 min. *D*, the activation of integrin αIIbβ3 induced by the indicated agonists was investigated. Whole blood from WT or CLEC-2 chimeras was diluted 15-fold with modified Tyrode's buffer. 25 μl of diluted whole blood was stimulated with the indicated platelet agonists for 5 min at room temperature, followed by the addition of FITC-conjugated control rat IgG or FITC-conjugated anti-activated mouse integrin αIIbβ3 (clone Jon-A) for 15 min at room temperature. Reactions were terminated by the addition of 400 μl of PBS, and the samples were then analyzed using a FACScan. Data are expressed as the mean of the median fluorescence intensity (*MFI*) S.E. ($n = 4–7$). *E*, CD62P expression stimulated by the indicated agonists was investigated. 25 μl of washed platelets (5×10^7/ml) was stimulated with the indicated platelet agonists for 5 min at room temperature, followed by the addition of PE-conjugated control rat IgG or PE-conjugated anti-mouse CD62P for 15 min at room temperature. Reactions were terminated by the addition of 400 μl of PBS, and the samples were then analyzed using a FACScan. Data are expressed as the mean of the median fluorescence intensity ± S.E. ($n = 4–7$). *F*, serotonin release from dense granules was investigated. Washed platelets (3×10^8/ml) were stimulated with the indicated platelet agonists for 5 min. After platelets were removed by centrifugation, the serotonin concentration of the supernatant was measured by enzyme-linked immunosorbent assay. Serotonin release is expressed as the percent serotonin concentration of the platelet lysate. Data are expressed as the mean ± S.E. ($n = 3$). *, $p < 0.05$; **, $p < 0.005$. *rhod*, rhodocytin; *col*, collagen; *rest*, resting.

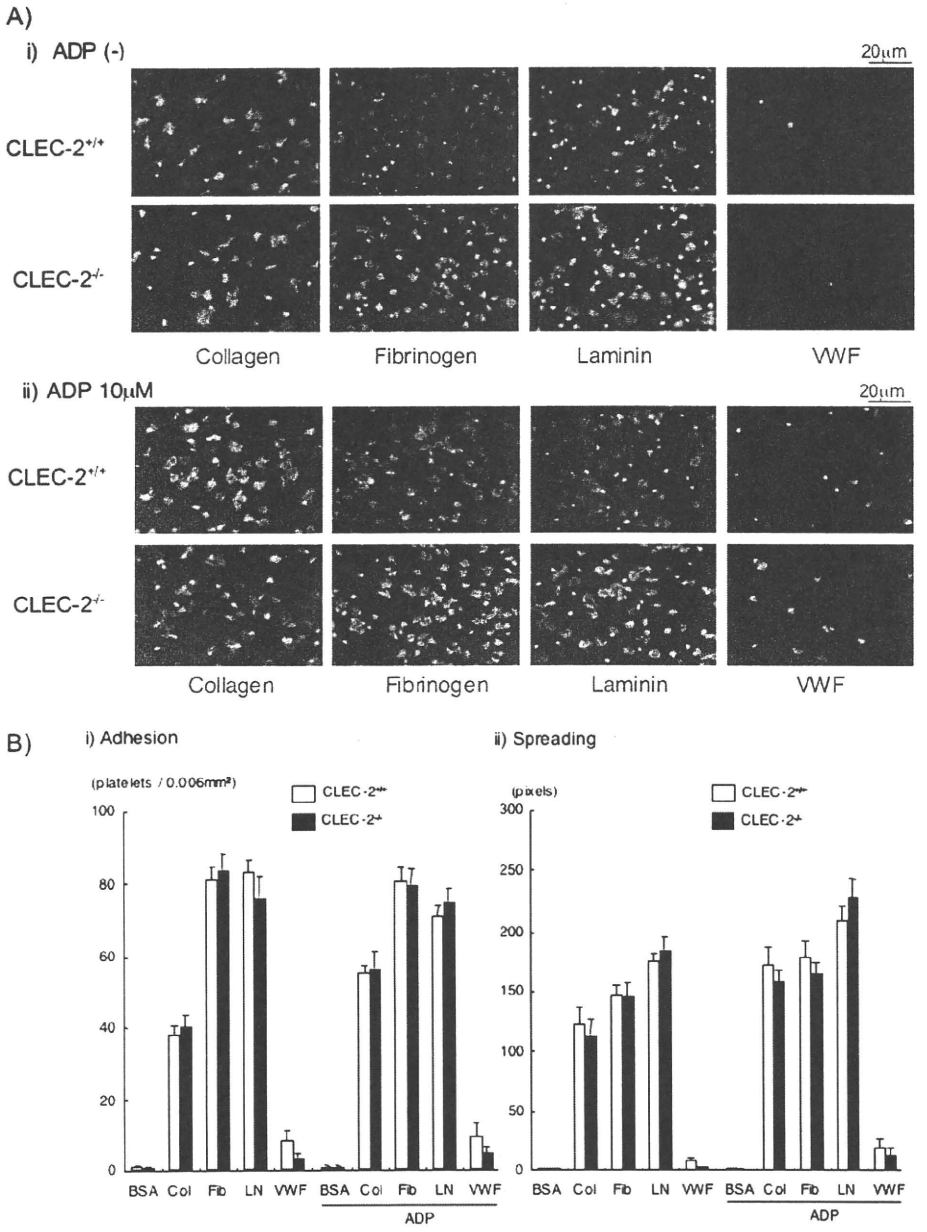


FIGURE 5. **CLEC-2-deficient platelets showed normal adhesion and spreading on the surface of collagen, fibrinogen, laminin, and vWF.** *A*, platelet spreading on the surface of major extracellular matrices was investigated. Washed platelets from WT chimeras (*Clec-2*$^{+/+}$) or CLEC-2 chimeras (*Clec-2*$^{-/-}$) were seeded on coverslips coated with laminin (*LN*), collagen (*Col*), fibrinogen (*Fib*), or vWF for 30 min at room temperature in the presence or absence of 10 μM ADP. Adherent platelets were fixed in 3% paraformaldehyde, permeabilized with 0.3% Triton X-100 for 5 min, and stained with TRITC-conjugated phalloidin. Platelets were visualized using an inverted fluorescence microscope and a digital camera. *B*, shown is the quantification of adherent platelets in the images in *A*. BSA-coated coverslips were prepared as a negative control. At least six images from two independent experiments were chosen at random per experiment and analyzed by two individuals, one of whom performed the analysis under blind conditions. Adherent platelets were counted (0.006 mm^2/image), and platelet surface area was analyzed using NIH Image.

tion between hCLEC-2-rFc2 molecules and that between mCLEC-2-rFc2 molecules were direct, with affinities of $(2.78 \pm 1.35) \times 10^{-7}$ and $(4.99 \pm 1.29) \times 10^{-7}$ M, respectively ($n = 4$) (Fig. 8*C*).

To further confirm the homophilic association of CLEC-2 under more physiological conditions, we investigated the platelet adhesion of WT or CLEC-2-deficient platelets on a surface coated with mCLEC-2-rFc2 or rFc2. To exclude the possibility that the Fc portion interacts with a stimulatory Fc receptor, FcγRIIA, we used murine platelets, which lack FcγRIIA. As shown in Fig. 9 (*A* and *B*), CLEC-2-deficient platelets adhered to collagen-coated surfaces to the same extent as WT platelets, whereas platelet adhesion to the surface of mCLEC-2-rFc2 was greatly and significantly inhibited with CLEC-2-deficient platelets ($p < 0.005$). These findings clearly suggest that CLEC-2 forms a homophilic association. Because CLEC-2-deficient platelets still adhered to mouse CLEC-2-coated surfaces and the adherent platelets showed spreading to some extent, it is likely that there is another CLEC-2 ligand on the surface of platelets.

We next investigated whether soluble recombinant CLEC-2 binds to the surface of suspended platelets by flow cytometry. As shown in Fig. 9*C*, the binding level of mCLEC-2-rFc2 to both WT and CLEC-2-deficient platelets was virtually the same as that of rFc2. However, there was a significant increase in mCLEC-2-rFc2 binding to both WT and CLEC-2-deficient platelets after platelet activation by PAR-4 peptides. However, it is important to note that the binding of mCLEC-2-rFc2 to activated WT platelets was significantly higher than that to activated CLEC-2-deficient platelets ($p < 0.05$). These findings suggest that CLEC-2 binds more avidly to CLEC-2 on the surface of activated platelets, but there appears to be another CLEC-2 ligand on the surface of activated platelets.

DISCUSSION

In this study, using CLEC-2-deficient mice for the first time, we have demonstrated that CLEC-2 is essential for blood/lymphatic vessel separation and thrombus formation *in vivo* through homophilic association. These findings imply that CLEC-2 is a potential target protein for the development of anti-platelet drugs and anti-metastatic drugs.

CLEC-2-deficient mice were lethal at the embryonic/neonatal stage with blood-filled, dilated, and tortuous lymphatic vessels that had anastomotic sites with blood vessels (blood/lym-

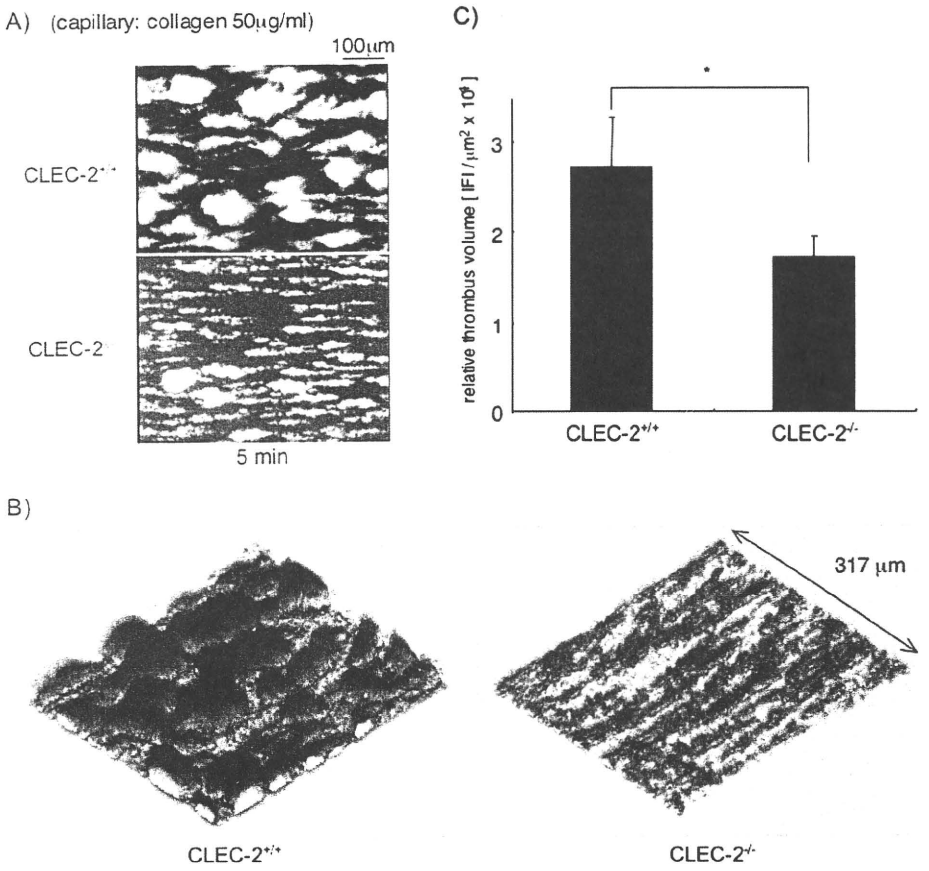


FIGURE 6. **Thrombus formation on the surface of collagen under flow conditions is impaired in CLEC-2 chimeras.** *A*, shown are video stills of thrombus formation on the surface of collagen under flow conditions. Capillary tubes were coated with 50 μg/ml collagen and blocked with PBS containing 2% BSA. Whole blood from WT chimeras (*Clec-2*$^{+/+}$) or CLEC-2 chimeras (*Clec-2*$^{-/-}$) anticoagulated with PPACK and heparin that had been pretreated with 3,3′-dihexyloxacarbocyanine iodide was perfused into capillaries at 2000 s^{-1}, and adherent platelets were visualized using a fluorescence video microscope. Movie data were converted into sequential photo images. *B*, shown are three-dimensional images of the thrombus formation. After perfusion of the blood, capillaries with thrombus were visualized using an Olympus FV-1000 confocal microscope. *C*, for measurement of thrombus volume, the images were analyzed using FluoView software, and the relative thrombus volume is expressed as integrated fluorescence intensity (*IFI*). *, $p < 0.05$ (n = at least 5 from two different mice).

phatic vessel misconnection). It is assumed that higher pressure in blood vessels drove blood components into lymphatic vessels through the anastomotic sites, resulting in dilated lymphatic vessels and retention of erythrocytes in the periphery. Progenitor cells of vascular and lymphatic endothelial cells express PECAM-1, and its expression in differentiated lymphatic endothelial cells is depressed compared with vascular endothelial cells. Although quantification has not been performed, we observed a higher level of PECAM-1 expression in the dilated lymphatic vessels in CLEC-2-deficient embryos than in WT embryos (Fig. 3, *A*, *D*, *G*, and *J*). It is plausible that blood flow from anastomosis may have led to high lymphatic pressure, which resulted in blood vessel endothelium-like change in lymphatic endothelial cells.

The mechanism by which CLEC-2 regulates blood/lymphatic vessel separation remains to be elucidated. Recently, Uhrin *et al.* (26) reported that platelet activation by podoplanin is a critical process for blood/lymphatic vessel separation. They found that platelet aggregates build up at the separation zone of podoplanin-positive lymph sacs and cardinal veins in WT embryos, but not in podoplanin-deficient embryos. Moreover, they proved that the same phenotypes are induced by anti-platelet drug, anti-podoplanin antibody, or inactivation of the *kindlin-3* gene required for platelet aggregation. We have found previously that podoplanin induces platelet aggregation through interaction with CLEC-2, and in this study, we demonstrated that CLEC-2 deficiency leads to poor blood/lymphatic vessel separation. Their findings combined with ours imply that platelet aggregates generated by CLEC-2/podoplanin interaction occlude the orifice of the lymph sacs, thereby inducing blood/lymphatic vessel separation. However, the phenotype of lymphatic vessels in podoplanin-deficient embryos is different from that in CLEC-2-deficient embryos; blood/lymphatic vessel misconnection disappears at postnatal days 10–14 in podoplanin-deficient mice, whereas the blood/lymphatic vessel misconnection is present persistently after E13.5 in CLEC-2-deficient mice. Although the majority of CLEC-2-deficient mice were lethal, we were able to obtain two adult CLEC-2-deficient mice, both of which showed the blood/lymphatic vessel misconnection phenotype (data not shown). The differences between podoplanin deficiency and CLEC-2 deficiency with regard to blood/lymphatic vessel misconnection suggest that there is a ligand (or ligands) other than podoplanin for CLEC-2 on the surface of lymphatic endothelial cells and that their interactions also play a role in blood/lymphatic vessel separation. CLEC-2 is expressed in platelets, megakaryocytes, and liver sinusoidal endothelial cells in humans and mice; however, it is also expressed in neutrophils in mice. Whether CLEC-2 in platelets, but not in neutrophils, regulates blood/lymphatic vessel separation is now under investigation using conditional knock-out mice that lack CLEC-2 only in platelets and megakaryocytes.

Although CLEC-2 plays a role in tumor metastasis, its powerful platelet-activating ability and specific expression in platelets and megakaryocytes imply that CLEC-2 also plays an important role in thrombosis and hemostasis. CLEC-2 knock-out mice should be utilized to accurately evaluate a role of CLEC-2 in thrombosis and hemostasis; however, we found that CLEC-2 knock-out mice are lethal at the embryonic/neonatal stage. We therefore generated irradiated chimeric animals that

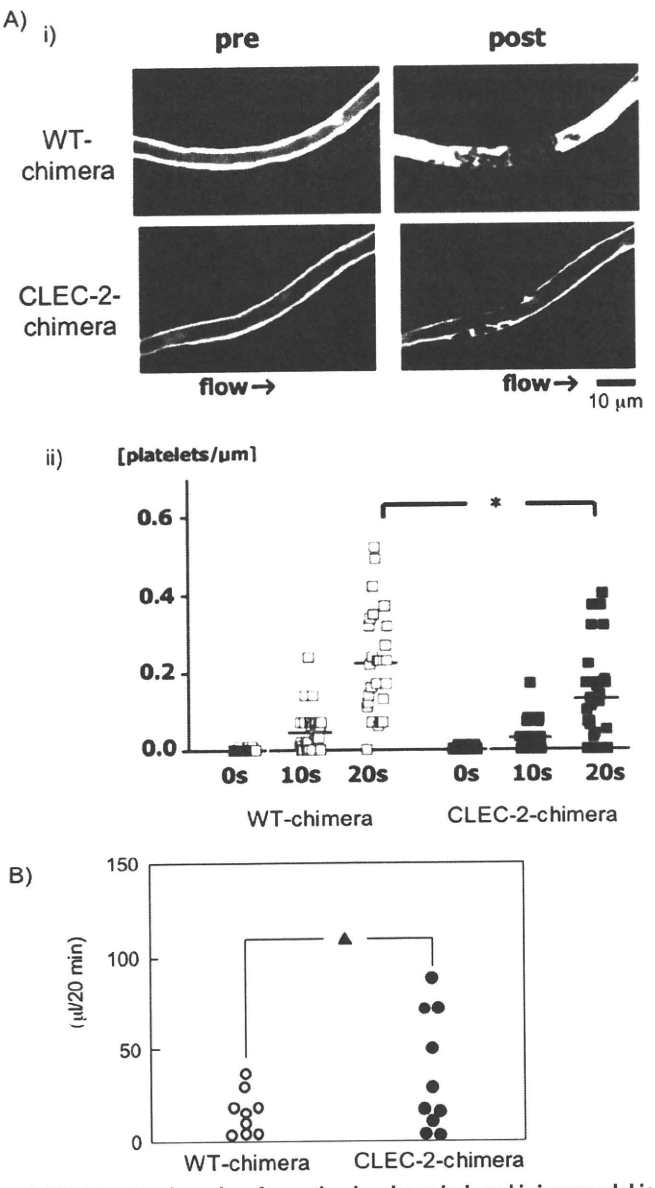
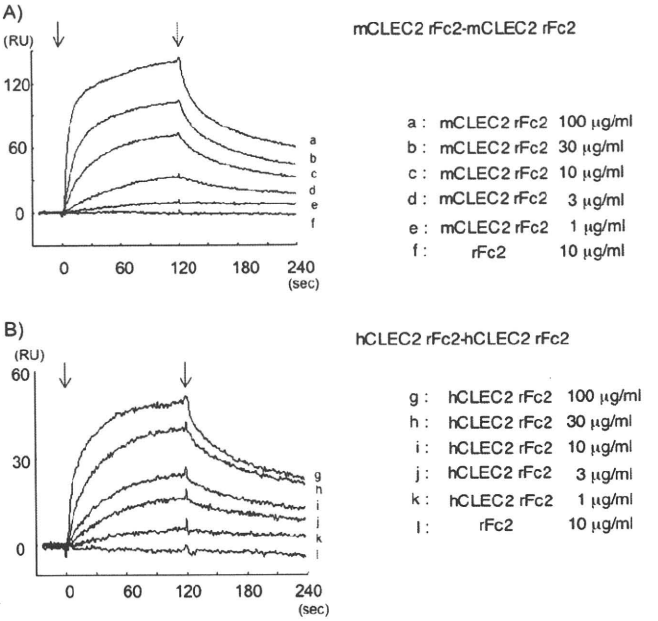


FIGURE 7. ***In vivo* thrombus formation in a laser-induced injury model is impaired in CLEC-2 chimeras without significant increase in tail bleeding.** *A*, video stills of mesenteric capillaries were obtained by intravital fluorescence microscopy before (*pre*) and 20 s after (*post*) laser-induced injury (*panel i*). The numbers of platelets in developing thrombi after laser injury to capillaries were calculated (*panel ii*). The *y axis* represents the numbers of platelets/micrometer of obtained vessel length. Results from WT and CLEC-2 chimeras 7 weeks after transplantation (17 weeks old) are shown ($n = 5$ each). *, $p < 0.05$. *B*, shown is the tail bleeding in WT and CLEC-2 chimeras. Each *symbol* represents one individual. Results from WT and CLEC-2 chimeras 8 weeks after transplantation (17 weeks old) are shown. ▲, not significant ($p = 0.08$).



C) Dissociation constants of CLEC2

interaction	Kd ($\times 10^{-7}$)
mCLEC2 with mCLEC2	4.99 ± 1.29
hCLEC2 with hCLEC2	2.78 ± 1.35

FIGURE 8. **CLEC-2 forms a homophilic association.** Different concentrations of hCLEC-2-rFc2 (*A*) or mCLEC-2-rFc2 (*B*) were flowed over an immobilized hCLEC-2-rFc2 (*A*), mCLEC-2-rFc2 (*B*), or control surface coated with rFc2. The *arrows* indicate the beginning and end of perfusion of hCLEC-2-rFc2 and mCLEC-2-rFc2. The results are shown from one experiment that is representative of four others. *RU*, resonance units. $K_d \pm$ S.E. ($n = 4$) of homophilic association of hCLEC-2-rFc2 or mCLEC-2-rFc2 was determined as described under "Experimental Procedures" (*C*).

had been rescued by transplantation of fetal liver taken from mice lacking CLEC-2. We observed significant inhibition of thrombus formation in CLEC-2 chimeras under flow conditions *in vivo* and *in vitro*, although platelet adhesion and spreading, which are the initial steps for thrombus formation, remained intact. Moreover, the bleeding tendency was minimal with CLEC-2 chimeras (Fig. 7*B*), suggesting that low molecular weight compounds acting as CLEC-2 antagonists are ideal candidates for a novel anti-platelet drug that inhibits pathological thrombus formation, but not physiological hemostasis. CLEC-2 deficiency leads to blood/lymphatic misconnections at the developmental stage; however, this does not seem to be a problem for a CLEC-2 antagonist, as an anti-platelet drug is normally administrated to adults.

Recently, May *et al.* (27) reported that anti-CLEC-2 antibody treatment of mice leads to the loss of CLEC-2 in circulating platelets for several days. These CLEC-2-deficient platelets displayed normal adhesion under flow conditions, but subsequent aggregate formation was severely impaired *in vitro* and *in vivo*, revealing an essential function of CLEC-2 in hemostasis and thrombosis. Although this unique study by May *et al.* shed light on the physiological role of CLEC-2, there still remains room for criticism of antibody-induced removal of antigen from platelets; it may have certain undesirable effects. Extensive antigen/antibody interaction on the platelet membrane may have some effects on thrombus formation. Alternatively, antibody-induced platelet activation and subsequent release of secondary mediators may result in desensitization of platelets. In mice, CLEC-2 is also expressed on peripheral blood neutrophils, and it mediates phagocytosis of antibody-coated beads and the production of proinflammatory cytokines, including tumor necrosis factor-α upon CLEC-2 stimulation (28). It is possible that the

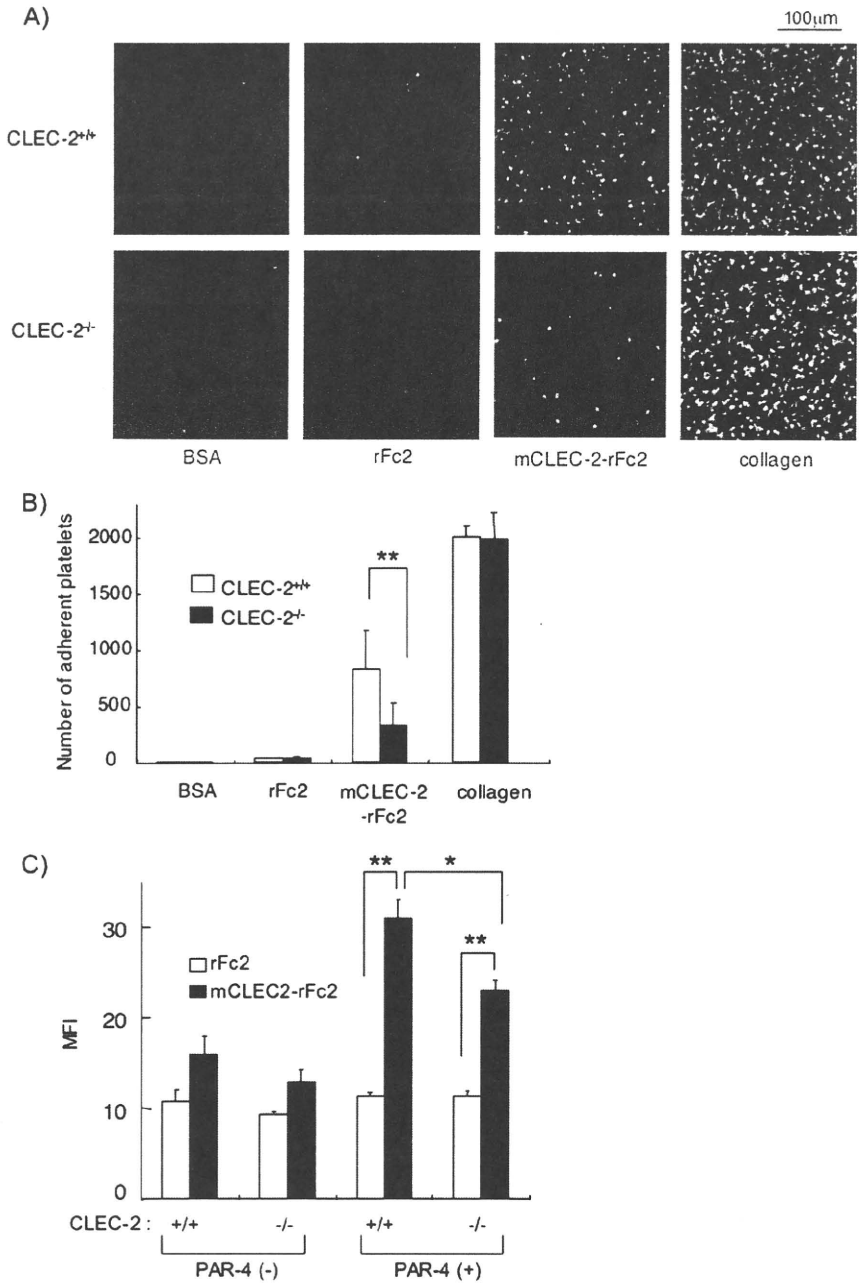


FIGURE 9. **Platelet adhesion to mCLEC-2-rFc2-coated surfaces and soluble mCLEC-2-rFc2 binding to platelets are inhibited in CLEC-2-deficient platelets.** *A*, shown is platelet adhesion on a surface coated with mCLEC-2-rFc2. Washed murine platelets from WT or CLEC-2 chimeras were seeded on coverslips coated with the indicated materials. Adherent platelets were fixed in paraformaldehyde, permeabilized with 0.3% Triton X-100, and stained with TRITC-conjugated phalloidin for 2 h. Platelets were visualized using an inverted fluorescence microscope and a digital camera. *B*, shown is the quantification of the platelet adhesion in *A*. At least six images from two independent experiments were chosen at random per experiment and analyzed by two individuals, one of whom performed the analysis under blind conditions. Adherent platelets were counted (0.006 mm^2/image). *C*, the binding of soluble mCLEC-2-rFc2 or rFc2 on the surface of WT (*Clec-2*$^{+/+}$) or CLEC-2-deficient (*Clec-2*$^{-/-}$) platelets was investigated by flow cytometry. Quantification of the soluble protein binding was performed using median fluorescence intensity (*MFI*). Data are expressed as mean ± S.E. ($n > 4$). Statistical significance was evaluated by Student's *t* test. In each case, *p* values <0.05 were taken as the minimum to indicate statistical significance. *, $p < 0.05$; **, $p < 0.005$.

anti-CLEC-2 antibody administered to mice may also work on CLEC-2 on neutrophils, thereby modifying cytokine generation and phagocytosis, which may affect thrombus formation under flow conditions or *in vivo*. In fact, it has been reported that tumor necrosis factor-α inhibits thrombus formation (29). Therefore, generation of CLEC-2-deficient mice has been awaited to solve these problems.

In our experiments, CLEC-2 chimeras showed only a mild increase in tail bleeding, which was not statistically significant compared with WT chimeras (Fig. 7*B*). In contrast to our findings, May *et al.* (27) reported that anti-CLEC-2 antibody-induced deficiency results in a marked increase in bleeding time: bleeding stopped in all of the control mice during a 20-min observation period (mean bleeding time of 6.1 ± 3.1 min), whereas bleeding continued for >20 min in 33% of the anti-CLEC-2 antibody-treated mice (mean bleeding time of 10.8 ± 6.0 min for those in which bleeding stopped). The discrepancy between gene-manipulated loss of antigen and antibody-induced loss has been reported previously; the bleeding time of GPVI/FcR γ-chain-deficient mice was not different from that of WT mice (30), whereas antibody-induced GPVI-deficient mice showed significantly increased bleeding times (158 ± 89 s in control mice *versus* 330 ± 103 s in antibody-treated mice) using the same protocol for the measurement of bleeding time. The idea of antibody-induced knock-out mice is derived from previously reported idiopathic thrombocytopenic purpura patients. Sugiyama *et al.* (31) and Moroi *et al.* (32) reported that idiopathic thrombocytopenic purpura patients contain an antibody against GPVI that stimulates platelet aggregation, leading to loss of GPVI from the platelet surfaces. These idiopathic thrombocytopenic purpura patients show a bleeding tendency, as is the case in antibody-induced GPVI- or CLEC-2-deficient mice, whereas genetically modified GPVI- or CLEC-2-deficient mice have no or less bleeding tendency. Although the precise mechanism for this discrepancy has not been elucidated, it is quite likely that antigen/antibody interaction induces some

unexpected biological responses, which may lead to overestimation of bleeding tendency due to loss of GPVI or CLEC-2.

Although it has been demonstrated that CLEC-2 is essential for thrombus formation, podoplanin, the only known internal ligand of CLEC-2 to date, is expressed in lymphatic endothelial cells or tumor cells and cannot be responsible for arterial thrombus formation. Thus, an important issue remains as to how CLEC-2 support thrombus formation under flow conditions *in vitro* and *in vivo*. From the findings of a previous work (27) and this study, it is suggested that the ligands of CLEC-2 are present in plasma or on the surface of activated platelets. We propose that CLEC-2 support thrombus formation, at least partly, through homophilic association based on the following findings. 1) Surface plasmon resonance detected homophilic association between recombinant CLEC-2 molecules. 2) Platelet adhesion onto a surface coated with recombinant CLEC-2 was significantly attenuated with CLEC-2-deficient platelets. 3) The binding of soluble recombinant CLEC-2 was significantly attenuated with CLEC-2-deficient platelets. We found that the interaction between hCLEC-2-rFc2 molecules is direct, with an affinity of 2.78×10^{-7} M, and that the affinity between mCLEC-2-rFc2 molecules is 4.99×10^{-7} M ($n = 4$). Christou *et al.* (5) reported that CLEC-2 and podoplanin interact directly, with an affinity of $(2.45 \pm 0.37) \times 10^{-5}$ M, which apparently suggests that the affinity between CLEC-2 and podoplanin is much weaker than the homophilic CLEC-2 interaction. One may argue that it is intriguing that podoplanin-expressing cells induce platelet aggregation, whereas platelets that express CLEC-2 with a high affinity homophilic interacting property do not form spontaneous aggregates. However, Christou *et al.* used monomeric recombinant CLEC-2 with a His tag and podoplanin-coated sensor chips, whereas we used dimeric recombinant CLEC-2 with Fc2, and the sensor chips were coated with this dimer form; we made dimeric CLEC-2, as it has been suggested that CLEC-2 exists as a dimer form on cell surfaces (22, 33). Because dimer forms of receptors are known to have much higher affinity for their ligands than single forms in the case of GPVI (34), this apparent discrepancy in the affinity of CLEC-2 for ligands may be due to the different experimental conditions. Alternatively, recombinant CLEC-2 fixed on the surface may assume a conformation that has higher homophilic activity than the natural CLEC-2 molecule on resting platelets, thereby giving a smaller K_d in a surface resonance study. This hypothesis may be extended to allege that CLEC-2 has two forms, one with relatively low homophilic activity in resting platelets and another with high homophilic activity in activated platelets. In support of this hypothesis, soluble CLEC-2 binding to suspended platelets was observed only after platelet activation (Fig. 9*C*). Moreover, surface expression of CLEC-2 was unaltered after stimulation (data not shown), suggesting higher homophilic binding activity of CLEC-2 in activated platelets. Additional experiments will need to address these issues.

We also assume that there are other CLEC-2 ligands on the surface of platelets than CLEC-2 itself. Although CLEC-2-deficient platelets showed reduced adhesion on the surface of recombinant CLEC-2 and reduced binding of soluble recombinant CLEC-2, it was not completely inhibited (Fig. 9). Moreover, CLEC-2-deficient platelets adhered to recombinant CLEC-2-coated surfaces showed spreading, suggesting that there is another ligand (or ligands) for CLEC-2 on the surface of platelets, the binding to which leads to platelet activation and spreading.

In conclusion, we have demonstrated, using CLEC-2-deficient mice, that CLEC-2 is essential for blood/lymphatic vessel separation and thrombus formation *in vivo* through homophilic association. We also suggest the possibility that there are other ligands for CLEC-2 that regulate lymphangiogenesis and thrombus formation. CLEC-2 is a promising target protein for the development of anti-platelet drugs that inhibit pathological thrombus formation, but not physiological hemostasis.

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Note Added in Proof—During the submission of our manuscript, Bertozzi *et al.* reported that platelets regulate lymphatic vascular development through CLEC-2/SLP-76 signaling (Bertozzi, C. C., Schmaier, A. A., Mericko, P., Hess, P. R., Zou, Z., Chen, M., Chen, C. Y., Xu, B., Lu, M. M., Zhou, D., Sebzda, E., Santore, M. T., Merianos, D. J., Stadtfeld, M., Flake, A. W., Graf, T., Skoda, R., Maltzman, J. S., Koretzky, G. A., and Kahn, M. L. (2010) *Blood*, in press).

REFERENCES

1. Cambi, A., and Figdor, C. G. (2003) *Curr. Opin. Cell Biol.* **15,** 539–546
2. Marshall, A. S., and Gordon, S. (2004) *Eur. J. Immunol.* **34,** 18–24
3. Suzuki-Inoue, K., Fuller, G. L., García, A., Eble, J. A., Pöhlmann, S., Inoue, O., Gartner, T. K., Hughan, S. C., Pearce, A. C., Laing, G. D., Theakston, R. D., Schweighoffer, E., Zitzmann, N., Morita, T., Tybulewicz, V. L., Ozaki, Y., and Watson, S. P. (2006) *Blood* **107,** 542–549
4. Suzuki-Inoue, K., Kato, Y., Inoue, O., Kaneko, M. K., Mishima, K., Yatomi, Y., Yamazaki, Y., Narimatsu, H., and Ozaki, Y. (2007) *J. Biol. Chem.* **282,** 25993–26001
5. Christou, C. M., Pearce, A. C., Watson, A. A., Mistry, A. R., Pollitt, A. Y., Fenton-May, A. E., Johnson, L. A., Jackson, D. G., Watson, S. P., and O'Callaghan, C. A. (2008) *Biochem. J.* **411,** 133–140
6. Schacht, V., Ramirez, M. I., Hong, Y. K., Hirakawa, S., Feng, D., Harvey, N., Williams, M., Dvorak, A. M., Dvorak, H. F., Oliver, G., and Detmar, M. (2003) *EMBO J.* **22,** 3546–3556
7. Kato, Y., Kaneko, M. K., Kunita, A., Ito, H., Kameyama, A., Ogasawara, S., Matsuura, N., Hasegawa, Y., Suzuki-Inoue, K., Inoue, O., Ozaki, Y., and Narimatsu, H. (2008) *Cancer Sci.* **99,** 54–61
8. Schacht, V., Dadras, S. S., Johnson, L. A., Jackson, D. G., Hong, Y. K., and Detmar, M. (2005) *Am. J. Pathol.* **166,** 913–921
9. Breiteneder-Geleff, S., Matsui, K., Soleiman, A., Meraner, P., Poczewski, H., Kalt, R., Schaffner, G., and Kerjaschki, D. (1997) *Am. J. Pathol.* **151,** 1141–1152
10. Abtahian, F., Guerriero, A., Sebzda, E., Lu, M. M., Zhou, R., Mocsai, A., Myers, E. E., Huang, B., Jackson, D. G., Ferrari, V. A., Tybulewicz, V., Lowell, C. A., Lepore, J. J., Koretzky, G. A., and Kahn, M. L. (2003) *Science* **299,** 247–251
11. Fu, J., Gerhardt, H., McDaniel, J. M., Xia, B., Liu, X., Ivanciu, L., Ny, A., Hermans, K., Silasi-Mansat, R., McGee, S., Nye, E., Ju, T., Ramirez, M. I., Carmeliet, P., Cummings, R. D., Lupu, F., and Xia, L. (2008) *J. Clin. Invest.* **118,** 3725–3737
12. Hirashima, M., Sano, K., Morisada, T., Murakami, K., Rossant, J., and Suda, T. (2008) *Dev. Biol.* **316,** 149–159
13. Turner, M., Mee, P. J., Costello, P. S., Williams, O., Price, A. A., Duddy, L. P., Furlong, M. T., Geahlen, R. L., and Tybulewicz, V. L. (1995) *Nature*

378, 298–302
14. Suzuki-Inoue, K., Inoue, O., Frampton, J., and Watson, S. P. (2003) *Blood* **102,** 1367–1373
15. Shin, Y., and Morita, T. (1998) *Biochem. Biophys. Res. Commun.* **245,** 741–745
16. Inoue, O., Suzuki-Inoue, K., Shinoda, D., Umeda, Y., Uchino, M., Takasaki, S., and Ozaki, Y. (2009) *FEBS Lett.* **583,** 81–87
17. Suzuki-Inoue, K., Yatomi, Y., Asazuma, N., Kainoh, M., Tanaka, T., Satoh, K., and Ozaki, Y. (2001) *Blood* **98,** 3708–3716
18. Law, D. A., DeGuzman, F. R., Heiser, P., Ministri-Madrid, K., Killeen, N., and Phillips, D. R. (1999) *Nature* **401,** 808–811
19. Nishimura, S., Manabe, I., Nagasaki, M., Seo, K., Yamashita, H., Hosoya, Y., Ohsugi, M., Tobe, K., Kadowaki, T., Nagai, R., and Sugiura, S. (2008) *J. Clin. Invest.* **118,** 710–721
20. Inoue, O., Suzuki-Inoue, K., McCarty, O. J., Moroi, M., Ruggeri, Z. M., Kunicki, T. J., Ozaki, Y., and Watson, S. P. (2006) *Blood* **107,** 1405–1412
21. Fuller, G. L., Williams, J. A., Tomlinson, M. G., Eble, J. A., Hanna, S. L., Pöhlmann, S., Suzuki-Inoue, K., Ozaki, Y., Watson, S. P., and Pearce, A. C. (2007) *J. Biol. Chem.* **282,** 12397–12409
22. Hughes, C. E., Pollitt, A. Y., Mori, J., Eble, J. A., Tomlinson, M. G., Hartwig, J. H., O'Callaghan, C. A., Futterer, K., and Watson, S. P. (2010) *Blood* **115,** 2947–2955
23. Ichise, H., Ichise, T., Ohtani, O., and Yoshida, N. (2009) *Development* **136,** 191–195
24. Nieswandt, B., and Watson, S. P. (2003) *Blood* **102,** 449–461
25. Takizawa, H., Nishimura, S., Takayama, N., Oda, A., Nishikii, H., Morita, Y., Kakinuma, S., Yamazaki, S., Okamura, S., Tamura, N., Goto, S., Sawaguchi, A., Manabe, I., Takatsu, K., Nakauchi, H., Takaki, S., and Eto, K. (2010) *J. Clin. Invest.* **120,** 179–190
26. Uhrin, P., Zaujec, J., Breuss, J. M., Olcaydu, D., Chrenek, P., Stockinger, H., Fuertbauer, E., Moser, M., Haiko, P., Fässler, R., Alitalo, K., Binder, B. R., and Kerjaschki, D. (2010) *Blood* **115,** 3997–4005
27. May, F., Hagedorn, I., Pleines, I., Bender, M., Vögtle, T., Eble, J., Elvers, M., and Nieswandt, B. (2009) *Blood* **114,** 3464–3472
28. Kerrigan, A. M., Dennehy, K. M., Mourão-Sá, D., Faro-Trindade, I., Willment, J. A., Taylor, P. R., Eble, J. A., Reis e Sousa, C., and Brown, G. D. (2009) *J. Immunol.* **182,** 4150–4157
29. Cambien, B., Bergmeier, W., Saffaripour, S., Mitchell, H. A., and Wagner, D. D. (2003) *J. Clin. Invest.* **112,** 1589–1596
30. Mangin, P., Yap, C. L., Nonne, C., Sturgeon, S. A., Goncalves, I., Yuan, Y., Schoenwaelder, S. M., Wright, C. E., Lanza, F., and Jackson, S. P. (2006) *Blood* **107,** 4346–4353
31. Sugiyama, T., Okuma, M., Ushikubi, F., Sensaki, S., Kanaji, K., and Uchino, H. (1987) *Blood* **69,** 1712–1720
32. Moroi, M., Jung, S. M., Okuma, M., and Shinmyozu, K. (1989) *J. Clin. Invest.* **84,** 1440–1445
33. Watson, A. A., Christou, C. M., James, J. R., Fenton-May, A. E., Moncayo, G. E., Mistry, A. R., Davis, S. J., Gilbert, R. J., Chakera, A., and O'Callaghan, C. A. (2009) *Biochemistry* **48,** 10988–10996
34. Miura, Y., Takahashi, T., Jung, S. M., and Moroi, M. (2002) *J. Biol. Chem.* **277,** 46197–46204

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Regular Article

Roles of Src-like adaptor protein 2 (SLAP-2) in GPVI-mediated platelet activation SLAP-2 and GPVI signaling

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ABSTRACT

Background: Glycoprotein VI (GPVI) /Fc receptor gamma (FcRγ)-chain complex is one of the collagen receptors in platelets and responsible for the majority of the intracellular signaling events through a similar pathway to immune receptors. Src-like adaptor protein 2 (SLAP-2) is a recently characterized adaptor protein predominantly expressed in hematopoietic cells. In T cells, SLAP-2 was reported to associate with several tyrosine phosphorylated proteins, and function as a negative regulator of signaling downstream of T cell antigen receptor by virtue of its interaction with the ubiquitin ligase c-Cbl. But the data regarding the presence and role of SLAP-2 proteins in platelets is limited.
Objectives: We describe the characterization of SLAP-2 in human platelets.
Methods: Human platelets were analyzed by Western blot analysis, immunoprecipitation, and pull down assay, etc.
Results: Immunoprecipitation revealed the presence of two forms of SLAP-2 with approximately 28kD and 25kD, and following stimulation of GPVI, the additional form with approximately 32kD apppeared. We have found that upon GPVI activation, SLAP-2 translocated from the Triton X-100-soluble fraction to the Triton X-100-insoluble cytoskeleton fraction, with concomitant association with Syk, c-Cbl, and LAT.
Conclusions: SLAP-2 appears to play a role in regulating signaling pathways by bringing important signaling molecules such as c-Cbl and Syk into proximity of cytoskeletal substrates. In platelets, SLAP-2 may have function as a negative regulator of GPVI-mediated signaling by interacting with c-Cbl, being similar to that reported in T cells.

Introduction

At sites of vascular injury, platelets come into contact with subendothelial collagen, which triggers their activation and the formation of a hemostatic plug. Collagen binds to at least two different receptors on the platelet membrane, one is $\alpha_2\beta_1$ integrin and the other is the immunoglobulin superfamily member glycoprotein VI (GPVI) [1]. The initial platelet contact with collagen and subsequent initiation of integrin activation are strictly dependent on functional GPVI [2,3]. GPVI is expressed exclusively in platelets and mature megakaryocytes, where it associates with the immunoreceptor tyrosine-based activation motif (ITAM)-containing transmembrane adaptor protein Fc receptor (FcR) γ-chain [4,5]. GPVI signals have the many similarities with that used by immune receptors, such as the T- and B-cell receptors on lymphocytes. Cross-linking of GPVI induces Src kinase-dependent tyrosine phosphorylation of the ITAM in the cytoplasmic tail of FcR γ-chain, leading to recruitment of the tyrosine kinase Syk [3]. Syk undergoes autophosphorylation and phosphorylation by Src family kinases Fyn and Lyn upon binding to the phosphorylated ITAM and initiates a downstream signaling cascade. Central to this signaling cascade is the formation of a signalosome that is composed of a series of adapter and effector proteins, which have been previously shown to participate in T cell receptor signaling. At the core of this signalosome is the transmembrane adapter LAT and the two cytosolic adapters SLP-76 and Gads [6]. These here proteins associate with a number of signaling molecules to regulate one of the major effecter enzymes in the GPVI signaling cascade, phospholipase C (PLC)γ2 and phosphoinositide (PI)3-kinase [7].

GPVI engagement activates several mechanisms that have been described to attenuate GPVI-mediated signaling. One of them is through regulation of the Cbl family of adaptor proteins [3]. c-Cbl is a ubiquitously expressed protein, initially characterized as an adaptor that functions as a negative regulator of both receptor and nonreceptor tyrosine kinases [8,9]. In addition to its adaptor function, c-Cbl also possesses a RING finger domain and has E3 ubiquitin ligase activity, which promotes the ubiquitination of activated tyrosine kinases [8,9]. Following TCR activation, c-Cbl is recruited to the

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activated TCR complex and tyrosine phosphorylated [8]. The activation of TCR signaling also leads to c-Cbl association with the Syk family kinases Syk and ZAP-70. The association between c-Cbl and the Syk family kinases results in a decrease in the activities and protein levels of these kinases [10,11], and thus in an overall down-regulation of signaling from the TCR. In platelets, c-Cbl is phosphorylated by CVX and collagen [12,13], and binds to several proteins that are involved in GPVI signaling, such as Src- and Syk-family protein tyrosine kinases and PI-3 kinase p85 subunit [12,14,15]. In c-Cbl-deficient mouse platelets, tyrosine phosphorylation of several proteins, including Syk, as a result of GPVI stimulation is enhanced as compared to that in wild-type platelets [12,14]. GPVI-induced aggregation is also enhanced in c-Cbl-null platelets [12,14]. Therefore, c-Cbl appears to play a negative regulatory role in platelets GPVI signal pathway as well.

Src-like adaptor protein 2 (SLAP-2) is a recently characterized adaptor protein bearing sequence and structural similarity to the Src-like adaptor protein (SLAP). SLAP-2 is predominantly expressed in hematopoietic cells [16–18]. SLAP-2 protein is composed of a N-terminal myristoylation consensus sequence that mediates SLAP-2 association with membranes, Src homology 3 (SH3) and SH2 domains, and a unique 78 amino acid C-terminal domain that is reported to bind c-Cbl [16–18]. Myristoylation of SLAP-2 facilitates its association with the plasma membrane and vesicles, while nonmyristoylated isoforms are found primarily within the cytosol [18]. In antigen receptor-stimulated cells, SLAP-2 associates with several tyrosine phosphorylated proteins, including the ubiquitin ligase Cbl, and has been demonstrated to function as a negative regulator of T and B-cell antigen receptor signaling by acting together with c-Cbl to mediate the ubiquitination and degradation of antigen receptor subunits and/or downstream effector molecules [17,18]. In recent studies, it was demonstrated that SLAP-2 is expressed in murine bone marrow-derived macrophages and plays a role in c-Cbl-dependent downregulation of the colony-stimulating factor-1 receptor (CSF-1R) signaling [19,20].

Little is known about SLAP-2 in platelets. Here, we provide the first report on the characterization of SLAP-2 in human platelets. We described that SLAP-2 was present on human platelets and had some modification and translocation following CVX stimulation. Furthermore, SLAP-2 was found to be capable of binding to c-Cbl, Syk and LAT in CVX-activated platelets. Our data suggest that SLAP-2 may play a role in negative regulation of GPVI-mediated signaling by interacting with c-Cbl in human platelets.

Materials and Methods

Materials

Polyclonal anti-SLAP-2 antibody, Syk kinase inhibitor Piceatannol and Src family kinase inhibitor PP2 was purchased from Calbiochem. Anti-phosphotyrosine monoclonal antibody 4G10, anti-LAT polyclonal antibody, and anti-ZAP-70 monoclonal antibody were purchased from Upstate Biotechnology. Anti-c-Cbl polyclonal antibody and anti-Syk monoclonal antibody were purchased from Santa Cruz Biotechnology. Anti-integrin β3 antibody was purchased from Chemicon International. Rabbit anti-mouse antibody, goat anti-rabbit antibody, and protein A conjugated to horseradish peroxidase (HRP) were purchased from Zymed laboratories and used to detect bound primary antibodies. The goat anti-mouse IgG labeled with Alexa Fluor 546 and the anti-rabbit IgG labeled with Alexa Fluor 488 were purchased from Invitrogen. The synthetic peptide consisting of the sequence Arg-Gly-Asp-Ser (RGDS), PI3-kinase inhibitor wortmannin, apyrase, prostaglandinE_1, $P2Y_1$ antagonist MRS2179, and thrombin was purchased from Sigma.

Preparation of human platelets

Blood was collected from drug-free healthy volunteers into one-nine volume of 130 mM sodium citrate. Platelet-rich plasma was obtained by centrifugation at 200×*g* for 20 min at room temperature, and platelets were isolated by centrifugation at 1000×*g* for 10 min in the presence of prostaglandinE_1 (0.1 μg/ml) at room temperature. Platelets were resuspended in a wash solution(6.85 mM citrate, 130 mM NaCl, 4 mM KCl, 5.5 mM glucose, pH 6.5) [21] in the presence of prostaglandinE_1 (0.1 μg/ml), recentrifuged at 1000×*g* for 10 min at room temperature, and resuspended in Hepes buffer (136 mM NaCl, 0.42 mM NaH_2PO_4, 2.7 mM KCl, 12 mM $NaHCO_3$, 5 mM Hepes, 5.5 mM glucose, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.2% bovine serum albumin (BSA;SIGMA), pH 7.4). Aliquots of platelets were stimulated with convulxin (CVX ;ALEXIS). In some experiments, as indicated, platelets were preincubated with inhibitors at 37 °C for 15 min prior to stimulation: 100 ng/ml of RGDS, 4U/ml of apyrase, 50 μM of piceatannol, 50 nM of wortmannin, and 20 μM of MRS2179.

Subcellular fraction of platelets

Triton X-100 insoluble cytoskeleton was isolated as described with the following modification [22]. Briefly, after platelets were stimulated for the indicated times, resting and stimlated platelets were immediately lysed by the addition of an equal volume of Triton lysis buffer (2% Triton X-100, 10 mM EGTA, 100 mM Tris-HCl, pH 7.4) containing protease inhibitors cocktail (Calbiochem), 2 mM phenylmethylsulfonyl fluoride (PMSF), and 2 mM sodium orthovanadate. After incubation on ice for 10 min, Triton X-100-insoluble residue was sedimented by centrifugation at 15000×*g* for 10 min at 4 °C, washed once, and homogenized with 1x sodium dodecyl sulfate (SDS) sample buffer (BioLabs) by boiling for 5 min (cytoskeleton-rich fraction). The Triton X-100-soluble fractions were solubilized in 3x SDS sample buffer by boiling for 5 min.

Immunoprecipitation

Platelets were lysed in NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 10 mM Tris-HCl, 2 mM EDTA, 1 mM EGTA, pH 7.5) containing protease inhibitors cocktail, 1 mM sodium orthovanadate, and 100 μM PMSF. After incubation on ice for 10 min, cell lysates were cleared by centrifugation at 20,000×*g* at 4 °C for 10 min and precleared by incubation with 50 μl of 50% protein A-Sepharose beads (Zymed) at 4 °C for 60 min. Precleared lysates were incubated with antibodies and protein A-Sepharose beads and then incubated at 4 °C with gentle rotation for 90 min or overnight. Immune complexes were washed four times in 1 ml of cold wash buffer (NP-40 lysis buffer containing 0.1% SDS), and bound proteins were eluted by boiling for 5 min in 3x SDS sample buffer. Phosphatase treatment was performed with 200 unit of lamda protein phosphatase (New England Biolabs) at 30 °C for 1 h.

Cloning of human SLAP-2 cDNA

Based on the sequence of human SLAP-2 [16], gene-specific primer were designed to PCR amplify the full-length human SLAP-2 cDNA (PCR primers: 5'-ATGGGAAGTCTGCCCAGCAG-3' (sense) and 5'-CTAGGCATCATCCAAAGAGA-3' (anti- sense). RT-PCR analysis was performed using first-Strand cDNA Synthesis kit (Amersham). We used $pd(N)_6$ primer and mRNA templates from human lymphocytes and platelets. The PCR reaction was performed in 50 μl volume containing 1 μl of cDNA, 5 μl of 10xPCR buffer, 0.2 mM dNTP, 1 μl of each primer, and 1.25 units of Taq polymerase (Applied Biosystems). The reaction mixture was denatured by heating at 96 °C for 5 min. Denaturation was followed by 40 cycles of 96 °C for 30 s, 60 °C for 1 min, 72 °C for 2 min, and final extension at 72 °C for 7 min. The PCR products were analyzed by agarose gel electrophoresis. The fragments were visualized by illumination after ethidium bromide staining.

In vitro binding assays

Full-length SLAP-2 cDNAs were cloned in frame into pGEX-4T1 (*Eco*RI/*Xho*I) in order to produce glutathione *S*-transferase (GST) fusion proteins in bacteria as described [18]. SLAP-2 expression constructs were confirmed by DNA sequencing. GST fusion proteins were produced in bacteria and purified on glutathione-Sepharose beads (Amersham). In vitro binding assays were done with platelets lysates either unstimulated or stimulated with CVX. Lysates were incubated with GST fusion proteins or GST alone for 90 min at 4 °C. Following several washes with cold wash buffer, bound proteins were eluted in 3x SDS sample buffer.

Western blotting

Eluted proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) on 8% or 12% gels under reduced conditions. Proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (BIO RAD) and incubated in a blocking solution of 5% (wt/vol) skim milk powder in 1x Tris-buffered saline with 0.05% Tween 20 (TBST) for a minimum of 30 min prior to the addition of antibodies. Blocked membranes were incubated with primary antibodies at room temperature for 1 h or at 4 °C overnight. Membranes were washed 3 times with TBST and incubated for 1 h with an appropriate secondary antibody conjugated to horseradish peroxidase. Membranes were washed an additional 3 times and bound antibodies were detected by using ECL Western blotting detection reagent (Amersham Bioscience).

Immunofluorescence

Platelets were fixed with 2% paraformaldehyde for 10 min at 4 °C. After fixation, platelets were permeabilized with 0.25% n-octyl-glucopyranoside for 15 min and washed in phosphate buffer saline (PBS, NaCl 58.5 mM, KCl 74.55 mM, KH_2PO_4 136 mM, Na_2HPO_4 358.4 mM, pH 7.4). Then, platelets for cytospin preparation (1.0×10^7 cells/slide) were deposited on glass slides using Auto Smear CF-12D (Sakura Finetek, Tokyo, Japan). Cytospin-prepared slides cells were blocked with PBS containing 3% BSA and 10% nomal goat IgG for 1 h, and were incubated with primary antibodies in 1% BSA-PBS for 1.5 h at 4 °C. After three washes with PBS, the appropriate secondary antibodies were added in 1% BSA-PBS for 30 min at room temperature. Anti-SLAP-2 antibody and anti-integrin β3 antibody were used at a 1:50 dilution, and the secondary antibodies, the goat anti-mouse IgG/Alexa Fluor 546 and the anti-rabbit IgG/Alexa Fluor 488, were used at 1:500. Cells were washed and mounted in Vectashield media (Vector Laboratories). Cover slips were then sealed with nail polish. Platelets were observed using a LSM-5 Pascal confocal laser microscope (Carl Zeiss Co).

Results

Expression of SLAP-2 in human platelets

Given the limited data regarding the presence and role of SLAP-2 proteins in platelets, initial experiments sought to determine which SLAP-2 were present in human platelets. For this, we performed reverse transcription (RT)-PCR in human platelets and lymphocytes as positive control. We were able to detect SLAP-2 transcript as an approximately 786 base pair PCR product in human platelets as well as lymphocytes (Fig. 1a, indicated by arrows). Next, to eliminate the possibility that lymphocytes were contaminated to RT-PCR assay, Western blot analyses were performed with a selective polyclonal antibody against human SLAP-2. In these experiments, whole cell lysates of Jurkat T cell was used as a control. In both platelets and Jurkat T cells, the anti-SLAP-2 antibodies specifically detected a protein doublet. The upper band migrated with an apparent molecular mass of 28 kDa (p28), and the smaller form of SLAP-2 migrated with an apparent molecular mass of 25 kDa (p25) (Fig. 1b, indicated by arrows). It was in agreement with a previous report that alternative translation initiation results in the expression of human SLAP-2 isoforms of approximately 28 and 25 kDa, respectively [23]. The smaller 25 kDa SLAP-2 isoform was reported that lacks both the amino-terminal myristoylation sequence and the serine-rich region present at the amino terminus of the long isoform [18,23]. p25 is expressed at low levels relative to p28 in platelets. (Fig. 1b) These data demonstrate that SLAP-2 is present in platelets.

SLAP-2 in resting platelets and CVX-stimulated platelets

Immunoprecipitation and Western blotting of human platelets with anti-SLAP-2 antibody revealed the presence of two bands with approximately 28 kDa and 25 kDa (Fig. 2a). Following stimulation with convulxin (CVX), a venom that acts by clustering GPVI, the additional higher-molecular-weight form with approximately 32 kDa (p32) was observed. This 32 kDa band was thought to be the same band

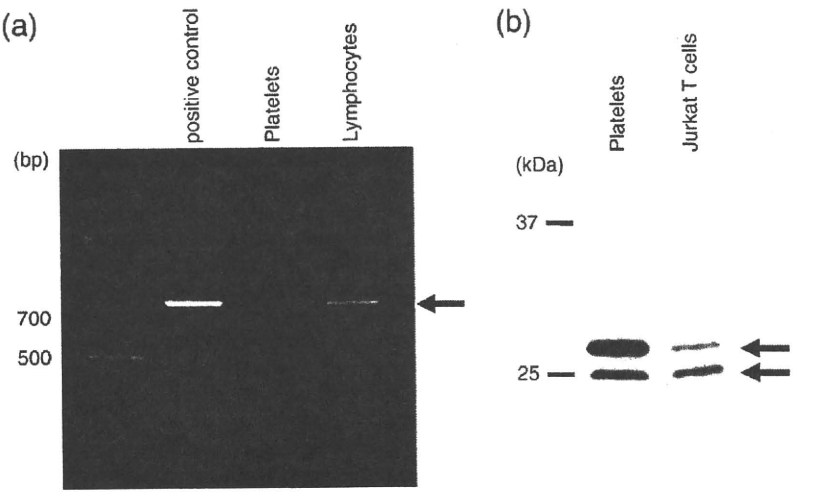

Fig. 1. SLAP-2 is expressed in platelets. (a) RT-PCR of SLAP-2 from purified human platelets and lymphocytes. Specific SLAP-2 primers were used in a standard PCR reaction with cDNA templates obtained by reverse transcription of mRNAs from platelets and lymphocytes. A 768 bp band corresponding to SLAP-2 transcript was detected (arrow). (b) Analysis of SLAP-2 protein expression by Western blotting. Human platelet lysate(approximately 15ug of protein) and Jurkat T cell lysate(20ug) were immunoblotted with anti-SLAP-2 antibody.

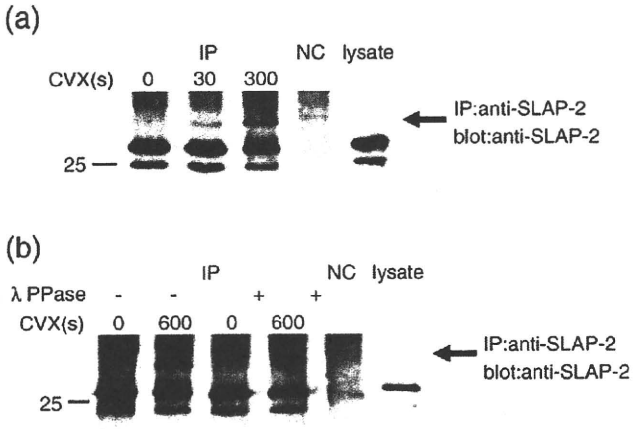

Fig. 2. p32 of SLAP-2 appeared in CVX-activated platelets. (a) Immunoprecipitates of SLAP-2 from human platelets. Lysates from platelets unstimulated(0) or stimulated with 100 ng/ml CVX for 30 seconds(30) or 5 minutes(300) were immunoprecipitated and immunoblotted with anti-SLAP-2 antibody. (b) Effect of phosphatase on modification of SLAP-2. Immunoprecipitates were treated with lamda protein phosphatase (λPPase)(+) or phosphatase buffer (-) at 30 °C for 30 minutes. Samples were Western blotted with anti-SLAP-2 antibody. IP: immunoprecipitation. NC: negative control. This experiment and those shown in all other figures are representive of three separate experiments.

that previously observed in COS cells transfected with SLAP-2 [18] or in bone marrow macrophages following CSF-1 stumulation [20], and arise from posttranslational modification. The 32 kDa band was lost when the immunoprecipitate was treated with lambda protein phosphatase (Fig. 2b) and was not detected by anti-phosphotyrosine antibodies (data not shown). These results suggest that phosphorylation on serine and/or threonine residues in amino terminus contribute to the formation of p32. In phosphoamino acid analysis of bone marrow macrophages, SLAP-2 was reported to be phosphorylated exclusively on serine residues [20]. But, we could not detect this band by Western blotting with anti-phosphothreonine and anti-phosphoserin antibodies. As described later, SLAP-2 is in association with c-Cbl in CVX-activated platelets. As c-Cbl has ubiquitin ligase activity [8], we investigated whether we could detect ubiquitination of p32 SLAP-2 in platelets by immunoprecipitating SLAP-2, followed by Western blotting for ubiquitin, using the anti-ubiquitin antibody P4D1 (Santa Cruz) and FK2 (Biomol). However, no ubiquitination of SLAP-2 could be detected in basal or GPVI-activated platelets. (data not shown)

Translocation of SLAP-2 from the Triton-soluble fraction to the cytoskeleton-rich fraction in platelets

Reorganization of the cytoskeleton and redistribution of platelet structural proteins and signaling molecules are thought to be important in early activation process of platelets. Since SLAP-2 contains an amino-terminal myristoylation sequence that mediates reversible association of SLAP-2 with plasma membrane, we investigated whether the subcellular localization of SLAP-2 changed following stimulation of platelets with CVX. The Triton-insoluble matrix of the platelet is operationally defined as the cytoskeleton, based on biochemical and morphological analysis [22,24]. To assess the detergent solubility of SLAP-2 in platelets, washed human platelets before and after activation with CVX were lysed with Triton X-100-containing buffer. The detergent-insoluble cytoskeleton fragments were precipitated by centrifugation at 15000×g. The immunoblots were probed with anti-SLAP-2 antibody (Fig. 3a). SLAP-2 sedimented at 15000×g from unstimulated platelets was very little. SLAP-2 was detected in the Triton-insoluble fraction 30 s after CVX stimulation, and the levels of SLAP-2 in this fraction increased throughout the time course of CVX treatment. There was a corresponding decrease in the amount of SLAP-2 that was detected in Triton-soluble fraction. Interestingly, isoforms of SLAP-2 did not behave together. The p32 isoform of SLAP-2 that appeared following CVX stimulation was detected mainly in Triton-insoluble cytoskeleton fraction, and the increase of p32 was parallel with the decrease of p28. While, the p25 isoform of SLAP-2 which lacks the myristoylation sequence in the cytoskeleton fraction little increased. These data suggests that p32 arises from modification of p28, but not p25, and that the sequence present only at p28 participates in translocation and modification of SLAP-2.

CVX induction of tyrosine phosphorylation of several platelet proteins was previously shown to be dependent on platelet aggregation mediated by the integrin receptor GPIIb-IIIa. To determine whether interaction of SLAP-2 and cytoskeleton proteins was dependent on GPIIb-IIIa receptor occupancy, platelets were activated by CVX in the presence of the RGDS peptide. This treatment significantly inhibited platelets aggregation and translocation of SLAP-2 (Fig. 3b). This result suggests that the association of SLAP-2 with the Triton-insoluble fraction is GPIIb-IIIa dependent. However, p32 was detected in the Triton-soluble fraction of platelets pretreated with RGDS peptide, suggesting that integrin outside-in signaling is not required for the CVX-mediated SLAP-2 modification. Moreover, SLAP-2 translocation and modification occurred in the presence of apyrase (4U/ml) and MRS2179 (P2Y1 antagonist, 20 μM), indicating that this process is not dependent upon the release of secondary mediators (data not shown).

To see whether inhibition of Src and Syk kinases affects platelet SLAP-2 redistribution, we pretreated platelets with PP2 and piceatannol. The inhibition of the kinases abolished the translocation of SLAP-2 into the cytoskeleton in CVX-activated platelets (data not shown). Inhibition of PI3-kinase by wortmannin also greatly decreased the SLAP-2 content in cytoskeleton in CVX-activated platelets . These treatment inhibited appearance of p32 SLAP-2. Therefore, it was thought that the translocation to cytoskeleton and modification of SLAP-2 were regulated downstream of these kinases.

An other platelet agonist was tested to determine if the translocation of SLAP-2 was downstream of their signaling cascades. SLAP-2 was detected in the Triton-insoluble fraction of thrombin-stimulated platelets, as shown in CVX-stimulated platelets (Fig. 3c). This translocation of SLAP-2 was inhibited in the presence of RGDS peptides (Fig. 3d). Thus, interaction of SLAP-2 with cytoskeleton proteins in thrombin-activated platelets was dependent on GPIIb-IIIa. The p32 isoform of SLAP-2 was detected in thrombin-stimulated platelets, and this was independent on GPIIb-IIIa.

We used immunofluorescence to investigate whether platelet activation might cause changes in SLAP-2 subcellular localization. Anti-integrin β3 stained by Alexa546 (red) was used as platelet surface marker. Platelets unstimulated or stimulated by CVX for 30 sec, 10 min, were fixed and stained for immunofluorescence. In resting platelets labeled with anti-SLAP-2 and stained by Alexa488 (green), fluorescence was diffused through the cytoplasm (Fig. 4). On the other hand, in activated platelets, SLAP-2 was predominantly localized near the plasma membrane. However, the merged images did not show marked yellow signals due to co-localization of the green and red signals, and we could not show the direct binding of SLAP-2 with plasma membrane (Fig. 4). These results confirmed the presence of SLAP-2 in human platelets and its translocation following CVX stimulation.

SLAP-2 associates with c-Cbl, Syk and LAT following GPVI cross-linking

It is known that platelet GPVI signals through a similar pathway to that used by the T cell antigen receptor (TCR) [2,3]. Then, SLAP-2 has been demonstrated to function as a negative regulator of TCR-mediated signaling by ability of its interaction with c-Cbl [16–18,23]. Since SLAP-2 is expressed in platelets, it is possible that it plays some roles in platelet GPVI signaling. In order to assess the ability of SLAP-2 to interact with

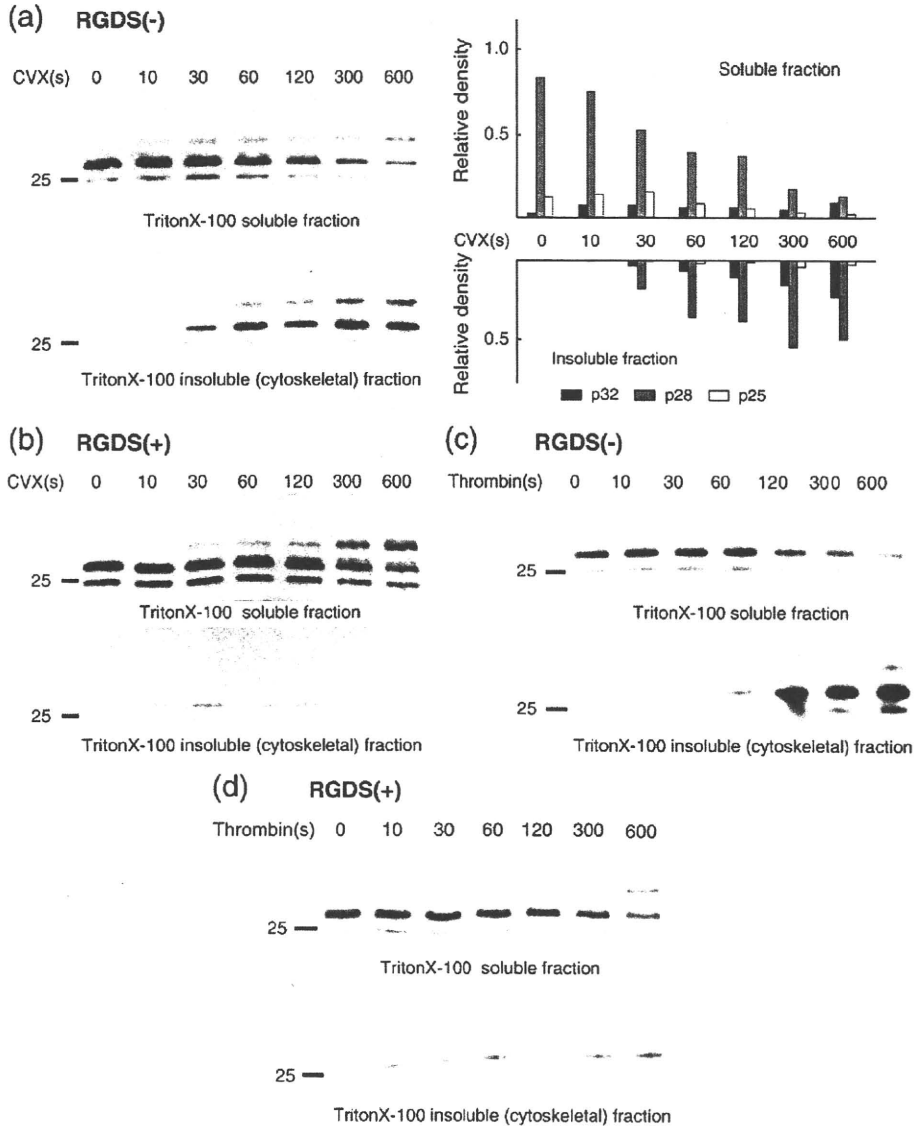

Fig. 3. Translocation of SLAP-2 to cytoskeleton in CVX-activated platelets requires GPIIb/IIIa. (a) Translocation of SLAP-2 to cytoskeleton following GPVI activation. Suspension of platelets (1.5×10^9 platelets/ml) were incubated with slight agitation in the presence of 100 ng/ml CVX for the indicated times (0-600 s). Incubations were terminated by addition of an equal volume of Triton X-100 lysis buffer. Lysates were centrifuged for 10 min at 15000×g. The sediment and supernatant were solubilized in an SDS sample buffer. Solubilized proteins were electrophoresed through SDS-PAGE and transferred to PVDF membrane. Blots were incubated with polyclonal antibodies against the SLAP-2. Densitometric analysis was shown at the right panel. (b) Effect of RGDS peptide on the translocation of SLAP-2 to the cytoskeleton. Platelets suspensions preincubated with 100 μg/ml RGDS peptide for 15 min were stimulated with 100 ng/ml CVX for the indicated times and then lysed in Triton X-100 lysis buffer. The sediment and supernatant were immnoblotted with anti-SLAP-2 antibodies. (c)(d) Suspension of platelets were incubated with slight agitation in the presence of 0.2U/ml thrombin for the indicated times (0-600 s). Triton-soluble and Triton-insoluble cytoskeleton fraction from thrombin-activated platelets untreated (c) or treated (d) with 100 μg/ml RGDS peptides were immunoblotted with anti-SLAP-2 antibodies.

tyrosine-phosphorylated signaling proteins downstream of the activated GPVI, we first tried to show interaction of endogenous SLAP-2 with other proteins by immunoprecipitation in several detergents, but we failed. We thought that the interaction between SLAP-2 with the other proteins is very weak, and detergents disrupted it. Then we made recombinant GST-SLAP-2 to test in vitro association with endogenous proteins in unstimulated or stimulated with CVX platelet lysates. SLAP-2 was interacted with tyrosine-phosphorylated proteins of approximately 120 kDa, 70 kDa and 36 kDa from CVX-stimulated human platelets (Fig. 5a). In T cell, SLAP-2 has been reported to associate with c-Cbl and ZAP-70 [17,18]. Therefore, we thought that the 120 kDa phosphoprotein represented c-Cbl, and the 70 kDa protein represented ZAP-70 or the related family member Syk. Subsequent immunoblotting with various antibodies identified which as c-Cbl, Syk and LAT, respectively (Fig. 5a). ZAP-70 was not detected. In addition, c-Cbl translocated from Triton X-100 soluble fraction to cytoskeletal fraction after CVX stimulation in similar manner of SLAP-2 (data not shown).

To investigate whether this interaction was truly due to stimulation through GPVI or whether it was caused by secondary agonist released from activated platelets, we used apyrase to prevent activation via ADP release. Inclusion of apyrase did not significantly affect the association of SLAP-2 with the above three proteins. Moreover, we examined the role of integrin outside-in signaling in SLAP-2 interaction. RGDS peptide, a selective blocker for integrins, did not affect the interaction of SLAP-2 with these proteins in CVX-stimulated platelets (Fig. 5b), where platelet aggregation was completely inhibited. These data suggest that CVX-induced GPVI clustering is primarily responsible for the interaction of SLAP-2 with

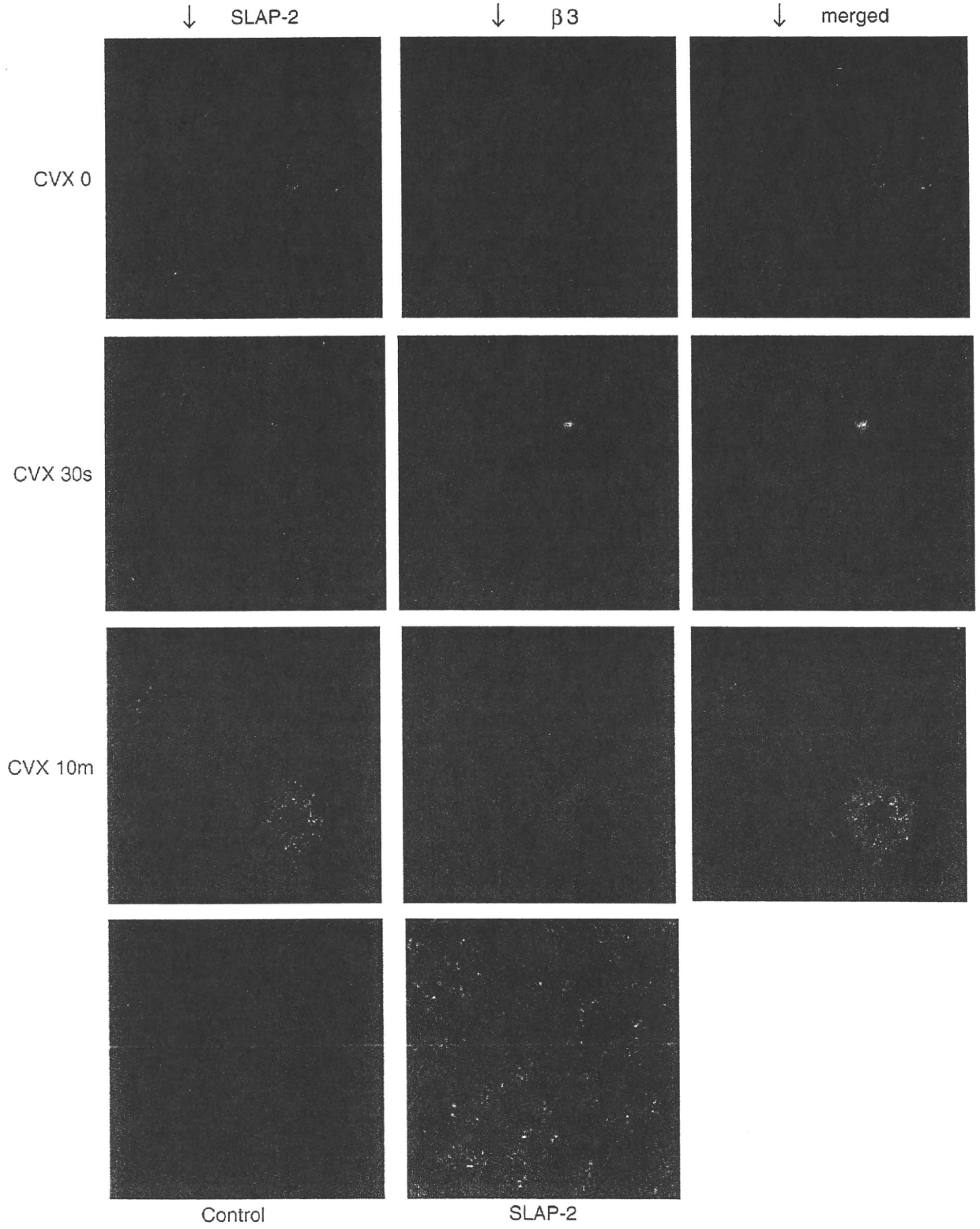

Fig. 4. Confocal experiments performed with resting and activated platelets. Platelets were immunostained for SLAP-2 and Alexa488 conjugated secondary antibodies (green, left panel). Platelet plasma membrane were stained with antibodies to integrin β3 and alexa546 conjugated secondary antibodies (red, middle panel). In the third panel, the merging of the respective images is shown.

these proteins, and that integrin outside-in signaling is not required for the CVX-mediated interaction of SLAP-2. Therefore, platelets were pretreated with RGDS and apyrase in the following experiments.

Inhibitor studies were performed to examine the interaction of SLAP-2 by CVX. To determine whether Src and Syk kinases are required for the interaction of SLAP-2 with c-Cbl, Syk and LAT, we used PP2 and

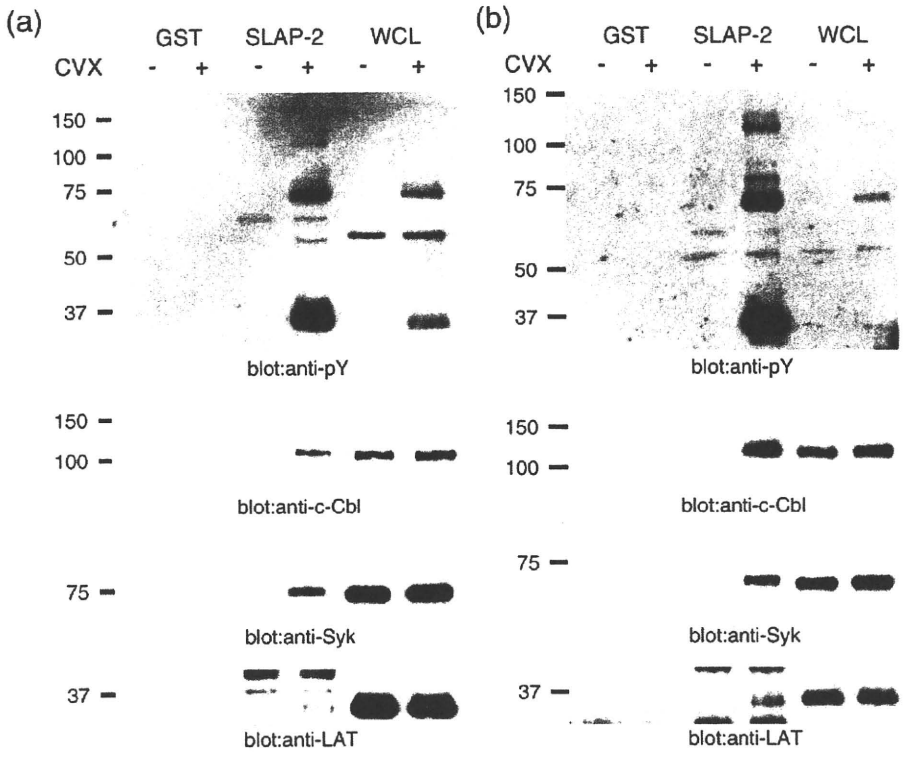

Fig. 5. SLAP-2 associates with tyrosine-phosphorylated c-Cbl, Syk and LAT following GPVI activation in platelets. (a) SLAP-2 associate with tyrosine-phospholylated proteins following GPVI activation. Purified immobilized GST fusion proteins with SLAP-2 were incubated with lysates from human platelets that were either unstimulated (-) or stimulated (+) with CVX. Immunoblotting was performed with the indicated antibodies. (b) Effect of apyrase and RGDS peptide on association of SLAP-2 with tyrosine phospholylated proteins. Lysates from platelets pretreated with 4U/ml apyrase and 100 μg/ml RGDS peptide for 15 minutes at 37 °C and unstimulated (-) or stimulated (+) with CVX were incubated with GST fusion protein with SLAP-2. Immunoblotting was performed with the indicated antibodies.

piceatannol to inhibit Src and Syk-kinases, respectively, where inhibition of platelet aggregation induced by CVX was observed (data not shown). PP2 completely inhibited both tyrosine phosphorylation of c-Cbl, Syk and LAT, and interaction of SLAP-2 with these proteins (Fig. 6a-d). PP2 also inhibited the p32 SLAP-2 production with CVX and thrombin stimulation (data not shown). On the other hand, Syk kinase inhibitor Piceatannol had no effect on interaction of SLAP-2 with these proteins. PI3-kinase plays a central role in the regulation of many cellular event, including recruitment of various proteins to cell membrane and regulation of the activity of a number of tyrosine kinases [3]. Pretreatment of platelets with wortmannin, which inactivates the catalytic p110 subunit of PI3-kinase, inhibited platelet aggregation induced by CVX [25]. However this treatment did not affect the interaction of SLAP-2 with c-Cbl, Syk and LAT. Interestingly, piceatanol and wortmannin inhibited the p32 SLAP-2 production with CVX stimulation and translocation to cytoskeletal fraction. These results demonstrated that association of SLAP-2 with these proteins through the GPVI activation is regulated predominantly downstream of Src-family kinases, and upstream of Syk kinases and PI3-kinase.

In order to determine whether interaction of SLAP-2 with these proteins is a general phenomenon downstream of all platelet agonist, we stimulated platelets with thrombin (Fig. 7). SLAP-2 associated with some tyrosine phosphorylated proteins. However, in contrast to GPVI-dependent agonists, immunoblotting experiment identified the proteins other than c-Cbl, Syk and LAT. This data suggest that thrombin does not cause the association of SLAP-2 with these proteins.

Discussion

In the present study, we have investigated the potential role of SLAP-2 in regulation of signaling by the GPVI in human platelets. Human SLAP-2 is predominantly expressed in leukocytes, lymph nodes, spleen, thymus, lung [16,17], and platelets (M.Tomlinson and S.P.W., July 2006, unpublished). We have now demonstrated that two protein isoforms of SLAP-2, p28 and p25, are expressed in human platelets. These isoforms are products of alternative translation initiation, the smaller isoform lacking both the myristoylation sequence and serine-rich region present at the amino terminus of the long isoform [18,23]. The p28 and p25 isoforms have been shown to be differentially localized to the membrane and cytoplasm, respectively [18]. The appearance of a higher molecular mass species of SLAP-2 (P32) following CVX stimulation suggested that SLAP-2 becomes phosphorylated in response to activation of GPVI. This idea was supported by the finding that protein phosphatase treatment of anti-SLAP-2 immunoprecipitates of SLAP-2 from CVX-stimulated platelets resulted in the disappearance of the P32 SLAP-2 and a concomitant increase of the smaller SLAP-2 species. Anti-phosphotyrosine western blotting of anti-SLAP-2 immunoprecipitates failed to reveal CVX-induced tyrosine phosphorylation of SLAP-2. This finding suggested that SLAP-2 became phosphorylated on serine and/or threonine residues in response to CVX stimulation. Anti-phosphoserine and phoshothreonin might not be sensitive enough to confirm this phosphorylation by Western blotting. A recent study by Loreto et al. [18] suggested that SLAP-2 undergoes phosphorylation when transiently expressed in COS cells. In addition, SLAP-2 is phosphorylated in serine residues [20] or tyrosine residues [19] in response to activation of the CSF-1 receptor when ectopically expressed in bone marrow macrophages or FD-Fms cells. However, this is the first report to describe receptor-induced phosphorylation of endogenous SLAP-2. The increase of p32 isoform that appeared following CVX stimulation was parallel with the decrease of p28, while the p25 isoform of SLAP-2 which lacks the myristoylation sequence slightly decreased. These

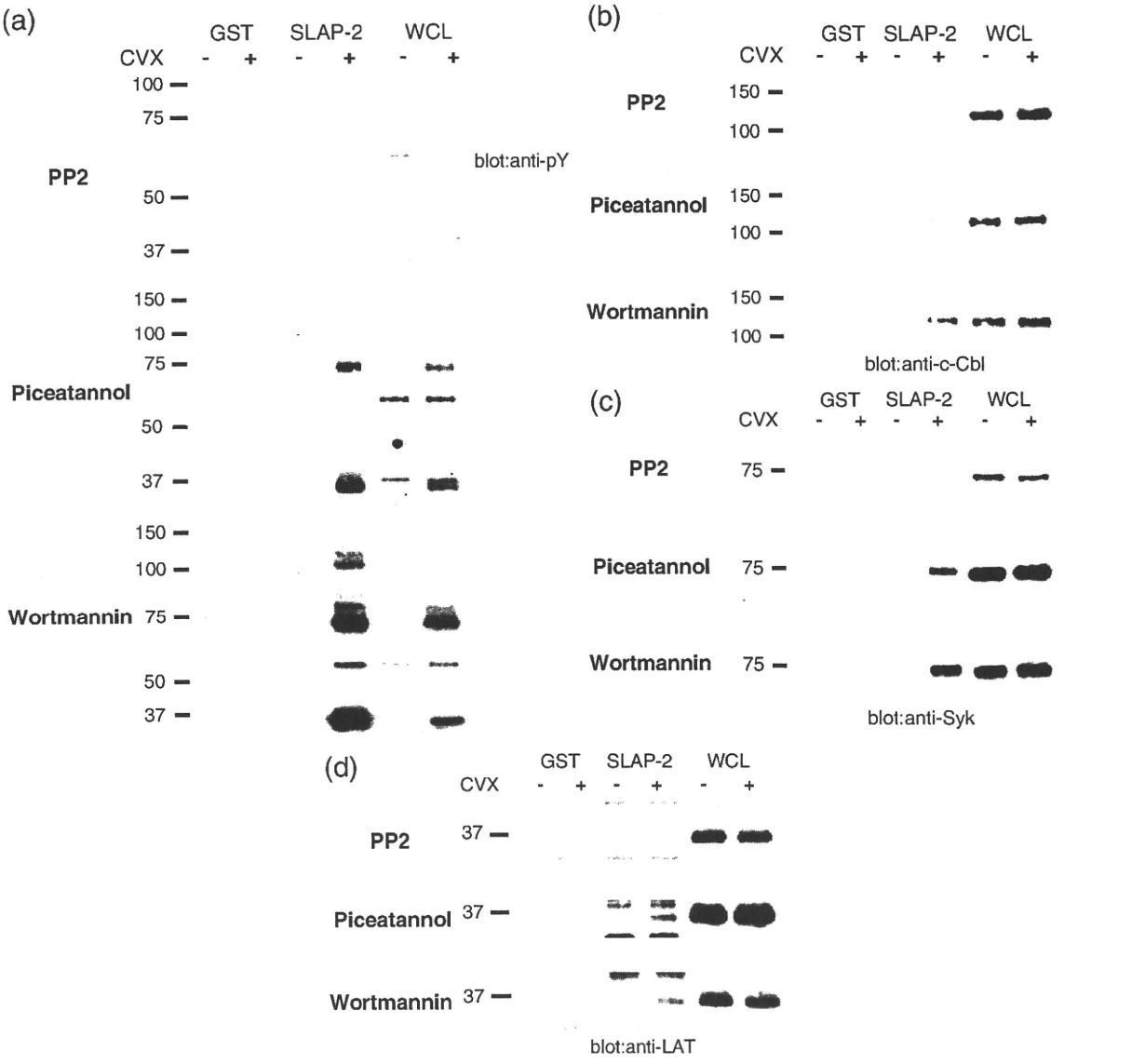

Fig. 6. Effect of inhibition of Src kinase, Syk kinase and PI3-kinase on association of SLAP-2 with c-Cbl, Syk and LAT. Washed platelets were treated with 100 ng/ml of CVX with or without PP2 (Src kinase inhibitor, 10 μM), piceatannol (Syk kinase inhibitor, 50 μM), and wortmannin (PI3-kinase inhibitor, 50nM) and incubated with GST fusion protein with SLAP-2. Immunoblotting was performed with the indicated antibodies. (a) anti-phosphotyrosine antibodies. (b) anti-c-Cbl antibodies. (c) anti-Syk antibodies. (d) anti-LAT antibodies.

data suggests that p32 arises from modification of p28, but not p25, and that the sequence present only at p28 is participates in phosphorylation of SLAP-2. It was in agreement with previous reports that the phosphorylation site in SLAP-2 was likely to reside within its N-terminus [18,20]. However, the increase of no less than 4 kDa of molecular mass could not be caused by phosphorylation alone. This phosphorylation may induce a conformational change in SLAP-2 that alters its electrophoretic mobility or trigger another modification of SLAP-2.

Various proteins in signal transduction pathways are myristoylated. In general, N-myristoylation is an irreversible protein modification that occurs co-translationally following removal of the initiator methionine residue by cellular methionylaminopeptidases [26]. N-myristoylation promotes weak and reversible protein-membrane and protein-protein interactions [27]. Many N-myristoylated proteins are membrane bound, and can be found in the plasma membrane or other intracellular membranes in eukaryotic cells. The binding energy provided by myristate is relatively weak and not sufficient to fully anchor a peptide or protein to a cellular membrane [27]. A second signal within the N-myristoylated protein is therefore required for efficient membrane binding [28]. This signal can be provided in several ways such as a polybasic domain or palmitate. A cluster of basic residues can provide electrostatic interactions with acidic membrane phospholipids. Palmitoylation is reversible, providing an additional hydrophobic interaction with the bilayer. Moreover, a conformational change of a protein regulates exposure of the myristate moiety. The transition between these two states is regulated by a mechanism known as the 'myristoyl switch'. Therefore myristoyl switch is mechanism for reversible membrane binding [28]. The presence of myristoylation sites within N-terminal of SLAP-2 suggests a potential mechanism for reversing its association with membranes. The proteins that are dually fatty acylated with myristate and palmitate nearly all contain the consensus sequence Met-Gly-Cys at their N-terminal [28]. SLAP-2 does not contain this palmitate motif, so the association of SLAP-2 with the plasma membrane is not regulated by palmitoylation. It is unclear whether the conformation of

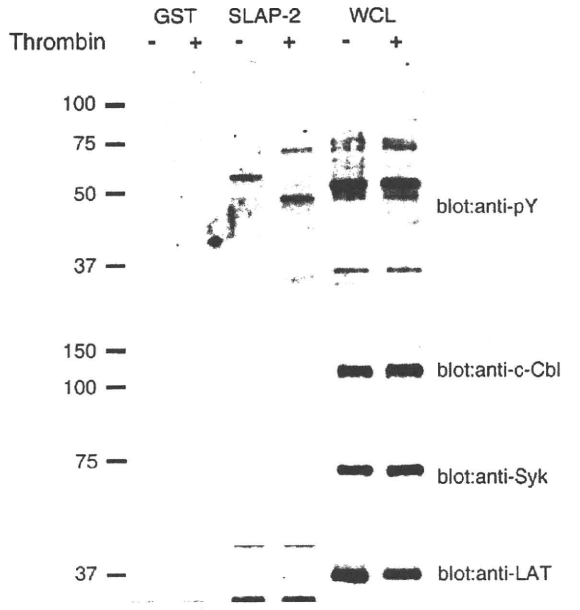

Fig. 7. SLAP-2 did not associate with c-Cbl, Syk and LAT in thrombin-actibated platelets. Purified immobilized GST fusion proteins with SLAP-2 were incubated with lysates from human platelets that were either unstimulated (-) or stimulated (+) with 0.2U/ml of thrombin. Immunoblotting was performed with the indicated antibodies.

SLAP-2 is changed, and therefore, it participates in the membrane binding or not. On the other hands, the N-terminus of SLAP-2 contains serine-rich region in positively charge. Phosphorylation of serine reduces the net charge of the basic cluster, altering its electrostatic potential and weakening its electrostatic binding to membranes containing acidic lipids. We showed that a fraction of SLAP-2 translocated to the Triton X-100-insoluble cytoskeletal fraction upon CVX stimulation of platelets. These data lead us to put forward the following hypothesis. In resting platelets, SLAP-2 exists mainly in cytoplasm. SLAP-2 can be attached to cytoskeleton with its myristate, but easily detached without second switch. Following activation of platelets by CVX stimulation, phosphorylation of the N-terminal serine works as a second switch, and results in redistribution of SLAP-2 to the Triton X-100-insoluble fraction(cytoskeleton). We could not detect the direct binding of SLAP-2 to actin by immunofluorescence study (data not shown), SLAP-2 may associate with some other cytoskeletal proteins or with actin indirectly. Upon activation, a lot of structural proteins and signaling molecules are recruited to the cytoskeleton [25,29–33]. Our results now show that SLAP-2 is also one of the components to associate to the cytoskeleton in response to CVX stimulation and facilitate signal transductions. Signaling by CVX involves rapid tyrosine phosphorylation of a number of proteins including Src, Syk and PI3-kinase [3]. The inhibitors of these molecules inhibited CVX-induced cytoskeletal translocation of SLAP-2. At the same time, these inhibitors inhibited CVX-induced phosphorylation of SLAP-2. These results are consistent with the hypothesis that phosphorylation of SLAP-2 results in translocation of SLAP-2 from the membrane to the cytoskeleton. However, RGDS blocked the association of SLAP-2 with the cytoskeleton in CVX-induced platelets, although it did not inhibit phosphorylation of SLAP-2. Thus, any step in the CVX signaling leading to GP IIb/IIIa activation is thought to be critical for CVX-induced interaction of SLAP-2 with cytoskeleton.

A function of SLAP-2 in lymphocytes is to negatively regulate antigen receptor signaling, most probably by facilitating the c-Cbl-directed ubiquitination of target proteins [16–18]. By analogy, SLAP-2 could potentially regulate GPVI signaling negatively by promoting the c-Cbl-directed ubiquitination of the GPVI and/or downstream signaling proteins. Consistent with studies in lymphocytes [18], our in vitro binding assays with GST fusion proteins of SLAP-2 revealed that SLAP-2 was capable of associating with c-Cbl and Syk, in place of Syk family tyrosine kinase ZAP-70, following GPVI stimulation. In T cells, ZAP-70 is tyrosine phosphorylated and binds to SLAP-2 following TCR stimulation [18]. In the process of c-Cbl-dependent downregulation of TCR, ZAP-70 associates with and is negatively regulated by c-Cbl [34]. In platelets, Syk is tyrosine phosphorylated, associated with c-Cbl and ubiquitinated in platelets activated with agonists that activate the GPVI dependent pathway [14]. The function of Syk in platelets may therefore be similar to that of ZAP-70 in T cell activation. On the other hand, we have observed an inducible association between SLAP-2 and transmembrane adaptor protein LAT, as was not previously reported in T cells [18], although the significance of this is unclear. Interestingly, in T cells, SLAP, but not SLAP-2, appears to inducibly associate with LAT following anti-CD3 stimulation [35,36]. The interaction of SLAP-2 and these proteins have several potential functions in cellular signaling. Adapter proteins form an intracellular scaffold that regulates and targets effector proteins to appropriate regions of the cell [3]. The function of the adaptor-cytoskeleton interaction is thought to be to bring the effector protein into close proximity to their molecular substrates that are involved in the signal events. Thus, if SLAP-2 is associated with components of the cytoskeleton in platelets, this might be important in bringing the effector protein into proximity with cytoskeletal substrates. Following stimulation of human platelets with CVX, SLAP-2 interacts with c-Cbl, Syk and LAT, and subsequently they translocate to the cytoskeleton. Therefore, SLAP-2 may play a role in promoting signaling pathway by bringing these signaling proteins to the cytoskeletal substrates.

We have reported in the present study that SLAP-2 is expressed in human platelets and has potentially functions as a novel component of a GPVI regulated signaling pathway. Indeed, activation of the GPVI induces phosphorylation of SLAP-2 and interaction with some signal proteins. Thus further studies that establish the specific role of SLAP-2 in signaling by the GPVI are required.

Conflict of interest statement

None of the authors have any conflicts of interest associated with this study.

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References

[1] Nieswandt B, Watson SP. Platelet-collagen interaction: is GPVI the central receptor? Blood 2003;102:449–61.
[2] Asazuma N, Wilde JI, Berlanga O, Leduc M, Leo A, Schweighoffer E, et al. Interaction of linker for activation of T cells with multiple adapter proteins in platelets activated by the glycoprotein VI-selective ligand, convulxin. J Biol Chem 2000;275:33427–34.
[3] Watson SP, Auger JM, McCarty OJ, Pearce AC. GPVI and integrin alphaIIb beta3 signaling in platelets. J Thromb Haemost 2005;3:1752–62.
[4] Gibbins JM, Okuma M, Farndale R, Barnes M, Watson SP. Glycoprotein VI is the collagen receptor in platelets which underlies tyrosine phosphorylation of the Fc receptor gamma-chain. FEBS Lett 1997;413:255–9.
[5] Berlanga O, Bobe R, Becker M, Murphy G, Leduc M, Bon C, et al. Expression of the collagen receptor glycoprotein VI during megakaryocyte differentiation. Blood 2000;96:2740–5.
[6] Watson SP, Asazuma N, Atkinson B, Berlanga O, Best D, Bobe R, et al. The role of ITAM- and ITIM-coupled receptors in platelet activation by collagen. Thromb Haemost 2001;86:276–88.
[7] Pasquet JM, Gross B, Quek L, Asazuma N, Zhang W, Sommers CL, et al. LAT is required for tyrosine phosphorylation of phospholipase cgamma2 and platelet activation by the collagen receptor GPVI. Mol Cell Biol 1999;19:8326–34.
[8] Swaminathan G, Tsygankov AY. The Cbl family proteins: ring leaders in regulation of cell signaling. J Cell Physiol 2006;209:21–43.

[9] Thien CB, Langdon WY. Cbl: many adaptations to regulate protein tyrosine kinases. Nat Rev Mol Cell Biol 2001;2:294–307.
[10] Murphy MA, Schnall RG, Venter DJ, Barnett L, Bertoncello I, Thien CB, et al. Tissue hyperplasia and enhanced T-cell signalling via ZAP-70 in c-Cbl-deficient mice. Mol Cell Biol 1998;18:4872–82.
[11] Rao N, Lupher Jr ML, Ota S, Reedquist KA, Druker BJ, Band H. The linker phosphorylation site Tyr292 mediates the negative regulatory effect of Cbl on ZAP-70 in T cells. J Immunol 2000;164:4616–26.
[12] Auger JM, Best D, Snell DC, Wilde JI, Watson SP. c-Cbl negatively regulates platelet activation by glycoprotein VI. J Thromb Haemost 2003;1:2419–26.
[13] Polgar J, Clemetson JM, Kehrel BE, Wiedemann M, Magnenat EM, Wells TN, et al. Platelet activation and signal transduction by convulxin, a C-type lectin from Crotalus durissus terrificus (tropical rattlesnake) venom via the p62/GPVI collagen receptor. J Biol Chem 1997;272:13576–83.
[14] Dangelmaier CA, Quinter PG, Jin J, Tsygankov AY, Kunapuli SP, Daniel JL. Rapid ubiquitination of Syk following GPVI activation in platelets. Blood 2005;105: 3918–24.
[15] Saci A, Rendu F, Bachelot-Loza C. Platelet alpha IIb-beta 3 integrin engagement induces the tyrosine phosphorylation of Cbl and its association with phosphoinositide 3-kinase and Syk. Biochem J 2000;3:669–76 351 Pt.
[16] Holland SJ, Liao XC, Mendenhall MK, Zhou X, Pardo J, Chu P, et al. Functional cloning of Src-like adapter protein-2 (SLAP-2), a novel inhibitor of antigen receptor signaling. J Exp Med 2001;194:1263–76.
[17] Pandey A, Ibarrola N, Kratchmarova I, Fernandez MM, Constantinescu SN, Ohara O, et al. A novel Src homology 2 domain-containing molecule, Src-like adapter protein-2 (SLAP-2), which negatively regulates T cell receptor signaling. J Biol Chem 2002;277:19131–8.
[18] Loreto MP, Berry DM, McGlade CJ. Functional cooperation between c-Cbl and Src-like adaptor protein 2 in the negative regulation of T-cell receptor signaling. Mol Cell Biol 2002;22:4241–55.
[19] Pakuts B, Debonneville C, Liontos LM, Loreto MP, McGlade CJ. The Src-like adaptor protein 2 regulates colony-stimulating factor-1 receptor signaling and down-regulation. J Biol Chem 2007;282:17953–63.
[20] Manes GA, Masendycz P, Nguyen T, Achuthan A, Dinh H, Hamilton JA, et al. A potential role for the Src-like adapter protein SLAP-2 in signaling by the colony stimulating factor-1 receptor. FEBS J 2006;273:1791–804.
[21] Jung SM, Moroi M. Signal-transducing mechanisms involved in activation of the platelet collagen receptor integrin alpha(2)beta(1). J Biol Chem 2000;275: 8016–26.
[22] Phillips DR, Jennings LK, Edwards HH. Identification of membrane proteins mediating the interaction of human platelets. J Cell Biol 1980;86:77–86.
[23] Loreto MP, McGlade CJ. Cloning and characterization of human Src-like adaptor protein 2 and a novel splice isoform, SLAP-2-v. Oncogene 2003;22:266–73.
[24] Fox JE, Boyles JK, Berndt MC, Steffen PK, Anderson LK. Identification of a membrane skeleton in platelets. J Cell Biol 1988;106:1525–38.
[25] Lu Q, Clemetson JM, Clemetson KJ. Translocation of GPIb and Fc receptor gamma-chain to cytoskeleton in mucetin-activated platelets. J Thromb Haemost 2005;3: 2065–76.
[26] Wolven A, Okamura H, Rosenblatt Y, Resh MD. Palmitoylation of p59fyn is reversible and sufficient for plasma membrane association. Mol Biol Cell 1997;8: 1159–73.
[27] Peitzsch RM, McLaughlin S. Binding of acylated peptides and fatty acids to phospholipid vesicles: pertinence to myristoylated proteins. Biochemistry 1993;32:10436–43.
[28] Resh MD. Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. Biochim Biophys Acta 1999;1451:1–16.
[29] Clark EA, Brugge JS. Redistribution of activated pp 60c-src to integrin-dependent cytoskeletal complexes in thrombin-stimulated platelets. Mol Cell Biol 1993;13: 1863–71.
[30] Fox JE. Cytoskeletal proteins and platelet signaling. Thromb Haemost 2001;86: 198–213.
[31] Tohyama Y, Yanagi S, Sada K, Yamamura H. Translocation of p72syk to the cytoskeleton in thrombin-stimulated platelets. J Biol Chem 1994;269:32796–9.
[32] Fox JE, Lipfert L, Clark EA, Reynolds CC, Austin CD, Brugge JS. On the role of the platelet membrane skeleton in mediating signal transduction. Association of GP IIb-IIIa, pp 60c-src, pp62c-yes, and the p21ras GTPase-activating protein with the membrane skeleton. J Biol Chem 1993;268:25973–84.
[33] Horvath AR, Muszbek L, Kellie S. Translocation of pp 60c-src to the cytoskeleton during platelet aggregation. EMBO J 1992;11:855–61.
[34] Naramura M, Kole HK, Hu RJ, Gu H. Altered thymic positive selection and intracellular signals in Cbl-deficient mice. Proc Natl Acad Sci USA 1998;95: 15547–52.
[35] Sosinowski T, Pandey A, Dixit VM, Weiss A. Src-like adaptor protein (SLAP) is a negative regulator of T cell receptor signaling. J Exp Med 2000;191:463–74.
[36] Tang J, Sawasdikosol S, Chang JH, Burakoff SJ. SLAP, a dimeric adapter protein, plays a functional role in T cell receptor signaling. Proc Natl Acad Sci USA 1999;96: 9775–80.

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RESEARCH ARTICLE Open Access

Natural killer cells control a T-helper 1 response in patients with Behçet's disease

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Abstract

Introduction: Behçet's disease (BD) is a multisystem inflammatory disorder, in which a T-helper 1 (Th1)-polarized immune response plays a major role in the pathogenic process. We evaluated the regulatory role of natural killer (NK) cells in Th1-biased immune responses in patients with BD.

Methods: We studied 47 patients with BD, including 10 with active disease (aBD) and 37 with inactive disease (iBD), and 29 healthy controls. The activation status and cytotoxic activity of NK cells were examined by flow cytometry. The levels of mRNAs for immune modulatory and cytotoxic molecules in NK cells were determined by quantitative PCR. The IL-12 signal strength in NK cells was determined by assessing the phosphorylation state of its downstream component, signal transducer and activator of transduction 4, by immunoblotting. Finally, NK cells' ability to modulate the Th1 response was evaluated by co-culturing NK cells and T cells without cell contact.

Results: $CD69^+$-activated NK cells were significantly increased in aBD compared with iBD or control samples, although their cytotoxic activities were similar. The iBD NK cells showed downregulated IL-12 receptor β_2 mRNA levels compared with aBD or control NK cells. The increased IL-13 expression was detected in a subset of BD patients: most of them had iBD. The IL-13 expression level in iBD patients was significantly higher than the level in controls, but was not statistically different compared with the level in aBD patients. The gene expression profile in iBD patients was consistent with the NK type 2 phenotype, and the shift to NK type 2 was associated with disease remission. NK cells from iBD patients showed impaired IL-12-induced signal transducer and activator of transduction 4 phosphorylation. Finally, iBD, but not control, NK cells suppressed IFNγ expression by aBD-derived $CD4^+$ T cells *in vitro*.

Conclusions: NK cells may control disease flare/remission in BD patients via NK type 2-mediated modulation of the Th1 response.

Introduction

Behçet's disease (BD) is a multisystem inflammatory disorder characterized by recurrent attacks of uveitis, genital ulcers, oral aphtoid lesions, and skin lesions such as erythema nodosum [1]. The etiology of BD remains unclear, but previous studies on the circulating $CD4^+$ T cells and affected lesions of BD patients with active disease showed elevated levels of T-helper 1 (Th1) cytokines, such as IFNγ and IL-12, indicating that a Th1-polarized immune response plays a major role in the pathogenic process [2-4]. In addition, we recently reported that cytotoxic lymphocytes, including $CD8^+$ and γδ T cells, are also involved in the pathogenesis of BD via their cytotoxic activity [5,6]. Natural killer (NK) cells are another lymphocyte subset with cytotoxic activity, but their reported numbers and cytotoxic activity in both circulation and BD-associated lesions have been inconsistent [7-9].

NK cells have long been regarded as an essential component of innate immunity, based on their nonspecific cytotoxic activity against virus-infected and tumor cells [10]. Recent evidence, however, indicates that NK cells also regulate innate and acquired immune responses through their secretion of soluble factors and/or cell-cell contact [11]. Recently, a classification of NK cells into two functional subsets based on their expression profiles of cytok-

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